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(71) Applicants:

- KIRIN BEER KABUSHIKI KAISHA
Chuo-Ku, Tokyo 104 (JP)
- MARINE BIOTECHNOLOGY INSTITUTE CO.,
LTD.
Bunkyo-ku, Tokyo 113 (JP)

(72) Inventors:

- MISAWA, Norihiko
Kiban Gijutsu Kenkyusho
Yokoma-shi Kanagawa 236 (JP)

• KONDO, Keiji

Kiban Gijutsu Kenkyusho
Kanazawa-ku, Yokohama-shi Kanagawa 236 (JP)

• KAJIWARA, Susumu

Kiban Gijutsu Kenkyusho
Kanazawa-ku, Yokohama-shi Kanagawa 236 (JP)

• YOKOYAMA, Akihiro

Shimizu Kenkyusho
Sodeshi-cho Shimizu-shi Shizuoka 424 (JP)

(74) Representative: Hansen, Bernd, Dr. Dipl.-Chem.
et al

Hoffmann, Eitle & Partner,
Patentanwälte,
Arabellastrasse 4
81925 München (DE)

(54) DNA CHAIN USEFUL FOR XANTHOPHYLL SYNTHESIS AND PROCESS FOR PRODUCING XANTHOPHYLLS

(57) The following DNA chains relate to xanthophylls having a keto group, represented by astaxanthin, and the following technique relates to a genetically engineered production of xanthophylls: a DNA chain having a base sequence coding for a polypeptide having an enzymatic activity of converting the 4-methylene group of β -ionone ring into a keto group; a DNA chain having a base sequence coding for a polypeptide having an enzymatic activity of converting the 4-methylene group of a 3-hydroxy- β -ionone ring into a keto group; a DNA chain having a base sequence coding for a polypeptide having an enzymatic activity of adding a hydroxyl group to the 3-carbon atom of a 4-keto- β -ionone ring; and a process for producing various xanthophylls, such as canthaxanthin and astaxanthin, by introducing the above DNA chain(s) into a suitable microorganism, e.g., *Escherichia coli*, followed by expression thereof.

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Description

Technical Field

The present invention relates to DNA strands useful for the synthesis of keto group-containing xanthophylls (keto-carotenoids) such as astaxanthin which are useful for heightening the color of cultured fishes and shellfishes such as sea breams, salmon, lobster and the like and is used for foods as a coloring agent and an antioxidant, and to a process for producing keto group-containing xanthophylls (ketocarotenoids) such as astaxanthin with use of a microorganism into which the DNA strands have been introduced.

Background Art

The term xanthophylls mean carotenoid pigments having an oxygen-containing group such as a hydroxyl group, a keto group or an epoxy group. Carotenoids are synthesized by the isoprenoid biosynthetic process which is used in common halfway with steroids and other terpenoids with mevalonic acid as a starting material. C15 farnesyl pyrophosphate (FPP) resulting from isoprene basic biosynthetic pathway is condensed with C5 isopentenyl pyrophosphate (IPP) to give C20 geranylgeranyl pyrophosphate (GGPP). Two molecules of GGPP are condensed to synthesize a colorless phytoene as an initial carotenoid. The phytoene is converted into phytofluene, ζ -carotene, neurosporene and then lycopene by a series of desaturation reactions, and lycopene is in turn converted into β -carotene by the cyclization reaction. It is believed that a variety of xanthophylls are synthesized by introducing a hydroxyl group or a keto group into the β -carotene (See Britton, G., "Biosynthesis of Carotenoids"; Plant Pigments, Goodwin, T.W. ed., London, Academic Press, 1988, pp. 133-182).

The present inventors have recently made it possible to clone a carotenoid biosynthesis gene cluster from an epiphytic non-photosynthetic bacterium *Erwinia uredovora* in *Escherichia coli* with an index of the yellow tone of the bacterium, a variety of combinations of the genes being expressed in microorganisms such as *Escherichia coli* to produce phytoene, lycopene, β -carotene, and zeaxanthin which is a derivative of β -carotene into which hydroxyl groups have been introduced (See Fig. 10; Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K., Harashima, K.; "Elucidation of the *Erwinia uredovora* Carotenoid biosynthetic Pathway by Functional Analysis of Gene Products Expressed in *Escherichia coli*", J. Bacteriol., 172, p.6704-6712, 1990; Misawa, N., Yamano, S., Ikenaga, H., "Production of β -carotene in *Zymomonas mobilis* and *Agrobacterium tumefaciens* by Introduction of the Biosynthesis Genes from *Erwinia uredovora*", Appl. environ. Microbiol., 57, p. 1847-1849, 1991; and Japanese Patent Application No. 58786/1991 (Japanese Patent Application No. 53255/1990): "DNA Strands useful for the Synthesis of Carotenoids").

On the other hand, astaxanthin, a red xanthophyll, is a typical animal carotenoid which occurs particularly in a wide variety of marine animals including red fishes such as a sea bream and a salmon, and crustaceans such as a crab and a lobster. In general, animals cannot biosynthesize carotenoids, so that it is necessary for them to ingest carotenoids synthesized by microorganisms or plants from their environments. Thus, astaxanthin has hitherto been used widely for strengthening the color of cultured fishes and shellfishes such as a sea bream, a salmon, a lobster and the like. Moreover, astaxanthin has attracted attention not only as a coloring matter in foods but also as an anti-oxidant for removing active oxygen generated in bodies, which causes carcinoma (see Takao Matsuno ed., "Physiological Functions and Bioactivities of Carotenoids in Animals", Kagaku to Seibutsu, 28, p. 219-227, 1990). As the sources of astaxanthin, there have been known crustaceans such as a krill in the Antarctic Ocean, cultured products of a yeast *Phaffia*, cultured products of a green alga *Haematococcus*, and products obtained by the organic synthetic methods. However, when crustaceans such as a krill in the Antarctic Ocean or the like are used, it requires laborious works and much expenses for the isolation of astaxanthin from contaminants such as lipids and the like during the harvesting and extraction of the krill. Moreover, in the case of the cultured product of the yeast *Phaffia*, a great deal of expenses are required for the gathering and extraction of astaxanthin, since the yeast has rigid cell walls and produces astaxanthin only in a low yield. Also, in the case of the cultured product of the green alga *Haematococcus*, not only a location for collecting sunlight or an investment of a culturing apparatus for supplying an artificial light is required in order to supply light which is essential to the synthesis of astaxanthin, but also it is difficult to separate astaxanthin from fatty acid esters as by-products or chlorophylls present in the cultured products. From these reasons, astaxanthin produced from biological sources is in the present situation inferior to that obtained by the organic synthetic methods on the basis of cost. The organic synthetic methods however have a problem of by-products produced during the reactions in consideration of its use as a feed for fishes and shellfishes and an additive to foods, and the products obtained by the organic synthetic methods are opposed to the consumer's preference for natural products. Thus, it has been desired to supply an inexpensive astaxanthin which is safe and produced from biological sources and thus has a good image to consumers and to develop a process for producing the astaxanthin.

Disclosure of the Invention

It would be considered very useful to find a group of genes for playing a role of the biosynthesis of astaxanthin, because it is possible to afford astaxanthin-producing ability to a microorganism optimum in safety as a food or in potentiality for producing astaxanthin, regardless of the presence of astaxanthin-producing ability, by introducing a gene cluster for astaxanthin biosynthesis into the microorganism. No problem of by-products as contaminants is caused in this case, so that it would be considered not so difficult to increase the production amount of astaxanthin with a recent advanced technique of gene manipulation to a level higher than that accomplished by the organic synthetic methods. However, the groups of genes for synthesizing zeaxanthin, one of the xanthophylls, have already been acquired by the present inventors as described above, while no genes encoding a keto group-introducing enzyme required for the synthesis of astaxanthin have not successfully obtained. The reason of the failure in obtaining the genes includes that the keto group-introducing enzyme is a membrane protein and loses its activity when isolated from the membrane, so that it was impossible to purify the enzyme or measure its activity and no information on the enzyme has been obtained. Thus, it has hitherto been impossible to produce astaxanthin in microorganisms by gene manipulation.

The object of the present invention is to provide DNA strands which contain genes required for producing keto group-containing xanthophylls (ketocarotenoids) such as astaxanthin in microorganisms by obtaining such genes coding for enzymes such as a keto group-introducing enzyme required for producing keto group-containing xanthophylls (ketocarotenoids) such as astaxanthin, and to provide a process for producing keto group-containing xanthophylls (ketocarotenoids) such as astaxanthin with the microorganisms into which the DNA strands have been introduced.

The gene cloning method which is often used usually comprising purifying the aimed protein, partially determining the amino acid sequence and obtaining genes by a synthetic probe cannot be employed because of the purification of the astaxanthin synthetic enzyme being impossible as described above. Thus, the present inventors have paid attention to the fact that the cluster of carotenoid synthesis genes in non-photosynthetic bacterium (*Erwinia*) functions in *Escherichia coli*, in which lycopene and β -carotene which are believed to be intermediates for biosynthesis of astaxanthin are allowed to produce with combinations of the genes from the gene cluster, and have used *Escherichia coli* as a host for cloning of astaxanthin synthetic genes. The present inventors have also paid attention to the facts that some marine bacteria have an astaxanthin-producing ability (Yokoyama, A., Izumida, H., Miki, W., "Marine bacteria produced astaxanthin", 10th International Symposium on Carotenoids, Abstract, CL11-3, 1993), that a series of related genes would constitute a cluster in the case of bacteria, and that the gene cluster would be expressed functionally in *Escherichia coli* in the case of bacteria. The present inventors have thus selected the marine bacteria as the gene sources. They have carried out researches with a combination of these two means and successfully obtained the gene group which is required for the biosynthesis of astaxanthin and the other keto group-containing xanthophylls from marine bacteria. They have thus accomplished the present invention. In addition, it has been first elucidated in the present invention that the astaxanthin synthesis gene cluster in marine bacteria constitutes a cluster and expresses its function in *Escherichia coli*, and these gene products can utilize β -carotene or lycopene as a substrate.

The DNA strands according to the present invention are set forth as follows.

- (1) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the β -ionone ring into a keto group.
- (2) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.
- (3) A DNA strand hybridizing the DNA strand described in (2) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (2).
- (4) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.
- (5) A DNA strand hybridizing the DNA strand described in (4) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (4).
- (6) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting β -carotene into canthaxanthin via echinenone and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.
- (7) A DNA strand hybridizing the DNA strand described in (6) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (6).
- (8) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting β -carotene into canthaxanthin via echinenone and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.
- (9) A DNA strand hybridizing the DNA strand described in (8) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (8).

(10) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the 3-hydroxy- β -ionone ring into a keto group.

(11) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the 3-hydroxy- β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.

(12) A DNA strand hybridizing the DNA strand described in (11) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (11).

(13) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the 3-hydroxy- β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.

(14) A DNA strand hybridizing the DNA strand described in (13) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (13).

(15) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting zeaxanthin into astaxanthin by way of 4-ketozeaxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.

(16) A DNA strand hybridizing the DNA strand described in (15) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (15).

(17) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting zeaxanthin into astaxanthin by way of 4-ketozeaxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.

(18) A DNA strand hybridizing the DNA strand described in (17) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (17).

(19) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for adding a hydroxyl group to the 3-carbon of the 4-keto- β -ionone ring.

(20) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for adding a hydroxyl group to position 3-carbon of the 4-keto- β -ionone ring and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 2.

(21) A DNA strand hybridizing the DNA strand described in (20) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (20).

(22) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for adding a hydroxyl group to position 3-carbon of the 4-keto- β -ionone ring and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 6.

(23) A DNA strand hybridizing the DNA strand described in (22) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (22).

(24) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting canthaxanthin into astaxanthin by way of phoenicoxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 2.

(25) A DNA strand hybridizing the DNA strand described in (24) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (24).

(26) A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting canthaxanthin into astaxanthin by way of phoenicoxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 6.

(27) A DNA strand hybridizing the DNA strand described in (26) and having a nucleotide sequence which encodes a polypeptide having an enzyme activity described in (26).

The present invention also relates to a process for producing xanthophylls.

That is, the process for producing xanthophylls according to the present invention is set forth below.

(1) A process for producing a xanthophyll comprising introducing the DNA strand described in any one of the above mentioned DNA strands (1) - (9) into a microorganism having a β -carotene-synthesizing ability, culturing the transformed microorganism in a culture medium, and obtaining canthaxanthin or echinenone from the cultured cells.

(2) A process for producing a xanthophyll comprising introducing the DNA strand described in any one of the above mentioned DNA strands (10) - (18) into a microorganism having a zeaxanthin-synthesizing ability, culturing the transformed microorganism in a culture medium, and obtaining astaxanthin or 4-ketozeaxanthin from the cultured cells.

(3) A process for producing a xanthophyll comprising introducing the DNA strand described in any one of the above mentioned DNA strands (19) - (27) into a microorganism having a canthaxanthin-synthesizing ability, culturing the transformed microorganism in a culture medium, and obtaining astaxanthin or phoenicoxanthin from the cultured cells.

(4) A process for producing a xanthophyll according to any one of the above mentioned processes (1) - (3), wherein the microorganism is a bacterium or yeast.

Brief Description of the Drawings

Fig. 1 illustrates diagrammatically the nucleotide sequence of the keto group-introducing enzyme gene (*crtW* gene) of the marine bacterium *Agrobacterium aurantiacus* sp. nov. MK1 and the amino acid sequence of a polypeptide to be encoded thereby.

Fig. 2 illustrates diagrammatically the nucleotide sequence of the hydroxyl group-introducing enzyme gene (*crtZ* gene) of the marine bacterium *Agrobacterium aurantiacus* sp. nov. MK1 and the amino acid sequence of a polypeptide to be encoded thereby.

Fig. 3 illustrates diagrammatically the nucleotide sequence of the lycopene-cyclizing enzyme gene (*crtY* gene) of the marine bacterium *Agrobacterium aurantiacus* sp. nov. MK1 and the amino acid sequence of a polypeptide to be encoded thereby.

Fig. 4 illustrates diagrammatically the continuation of the sequences following to those illustrated in Fig. 3.

Fig. 5 illustrates diagrammatically the nucleotide sequence of the xanthophyll synthesis gene cluster of the marine bacterium *Agrobacterium aurantiacus* sp. nov. MK1.

The letters A - F in Fig. 5 correspond to those in Figs. 1 - 4.

Fig. 6 illustrates diagrammatically the continuation of the sequence following to that illustrated in Fig. 5.

Fig. 7 illustrates diagrammatically the continuation of the sequence following to that illustrated in Fig. 6.

Fig. 8 illustrates diagrammatically the continuation of the sequence following to that illustrated in Fig. 7.

Fig. 9 illustrates diagrammatically the continuation of the sequence following to that illustrated in Fig. 8.

Fig. 10 illustrates diagrammatically the carotenoid biosynthetic route of the non-photosynthesis bacterium *Erwinia uredovora* and the functions of the carotenoid synthetic genes.

Fig. 11 illustrates diagrammatically the main xanthophyll biosynthetic routes of marine bacteria *Agrobacterium aurantiacus* sp. nov. MK1 and *Alcaligenes* sp. PC-1 and the functions of the xanthophyll synthesis genes.

The function of *crtY* gene, however, has been confirmed only in the former bacterium.

Fig. 12 illustrates diagrammatically a variety of deletion plasmids containing the xanthophyll synthesis genes (cluster) of the marine bacterium *Agrobacterium aurantiacus* sp. nov. MK1.

The letter P represents the promoter of the *lac* of the vector pBluescript II SK. The positions of cutting with restriction enzymes are represented by abbreviations as follows: Sa, *SacI*; X, *XbaI*; B, *BamHI*; P, *PstI*; E, *EcoRI*; S, *SalI*; A, *Apal*; K, *KpnI*; St, *StuI*; N, *NruI*; Bg, *BglII*; Nc, *NcoI*; Hc, *HincII*.

Fig. 13 illustrates diagrammatically the nucleotide sequence of the keto group-introducing enzyme gene (*crtW* gene) of the marine bacterium *Alcaligenes* sp. PC-1 and the amino acid sequence of a polypeptide to be encoded thereby.

Fig. 14 illustrates diagrammatically the continuation of the sequences following to those illustrated in Fig. 13.

Fig. 15 illustrates diagrammatically the nucleotide sequence of the hydroxyl group-introducing enzyme gene (*crtZ* gene) of the marine bacterium *Alcaligenes* sp. PC-1 and the amino acid sequence of a polypeptide to be encoded thereby.

Fig. 16 illustrates diagrammatically the nucleotide sequence of the xanthophyll synthetic gene cluster of the marine bacterium *Alcaligenes* sp. PC-1 and the amino acid sequence of a polypeptide to be encoded thereby. The letters A - D in Fig. 16 correspond to those in Figs. 13 - 15.

Fig. 17 illustrates diagrammatically the continuation of the sequences following to those illustrated in Fig. 16.

Fig. 18 illustrates diagrammatically the continuation of the sequences following to those illustrated in Fig. 17.

Fig. 19 illustrates diagrammatically a variety of deletion plasmids containing the xanthophyll synthetic genes (cluster) of the marine bacterium *Alcaligenes* sp. PC-1.

The letter P represents the promoter of the *lac* of the vector pBluescript II SK+.

Fig. 20 illustrates diagrammatically xanthophyll biosynthetic routes containing minor biosynthetic routes in the marine bacteria *Agrobacterium aurantiacus* sp. no. MK1 and *Alcaligenes* sp. PC-1 and the functions of the xanthophyll synthesis genes.

Minor biosynthetic routes are represented by dotted arrows.

Best Mode for carrying out the Invention

The present invention is intended to provide DNA strands which are useful for synthesizing a keto group-containing xanthophylls (ketocarotenoids) such as astaxanthin derived from a marine bacteria *Agrobacterium aurantiacus* sp. nov. MK1 and *Alcaligenes* sp. PC-1, and a process for producing keto group-containing xanthophylls (ketocarotenoids), i.e. astaxanthin, phoenicoxanthin, 4-ketozeaxanthin, canthaxanthin, and echinenone with use of a microorganism into which the DNA strands have been introduced.

The DNA strands according to the present invention are in principle illustrated generally by the aforementioned DNA strands (1), (10) and (19) from the standpoint of the fine chemical-generating reaction, and basically defined by the aforementioned DNA strands (2), (4), (11), (13), (20) and (22). The specific examples of the DNA strands (2) and (4) are the aforementioned DNA strands (6) and (8); the specific examples of the DNA strands (11) and (13) are the aforementioned DNA strands (15) and (17); and the specific examples of the DNA strands (20) and (22) are the aforementioned DNA strands (24) and (26). In this connection, the DNA strands (3), (5), (7), (9), (12), (14), (16), (18), (21), (23), (25) and (27) hybridize the DNA strands (2), (4), (6), (8), (11), (13), (15), (17), (20), (22), (24) and (26), respectively, under a stringent condition.

The polypeptides encoded by the DNA strands according to the present invention have amino acid sequences substantially in a specific range as described above in SEQ ID NOS: 1 - 2, and 5 - 6 (Figs. 1 - 2, and 13 - 15), e.g. an amino acid sequence of amino acid Nos. 1 - 212 in SEQ ID NOS: 1 (A - B in Fig. 1). In the present invention, four polypeptides encoded by these DNA strands, that is four enzymes participating in the xanthophyll-producing reaction) may be modified by deletion, substitution or addition in some of the amino acids provided that the polypeptides have the enzyme activities as described above (see Example 13). This corresponds to that "amino acid sequencessubstantially ...". For instance, an enzyme of which amino acid at the first position (Met) has been deleted is also involved in the polypeptide or enzyme obtained by the modification of the amino acid sequence. In this connection, it is needless to say that the DNA strands according to the present invention for encoding the polypeptides also include, in addition to those having nucleotide sequences in a specific range shown in SEQ ID NOS: 1 - 2, and 13 - 15 (Figs. 1 - 2, and 13 - 15), degenerate isomers encoding the same polypeptides as above except degenerate codons.

Keto group-introducing enzyme gene (crtW)

The DNA strands (1) - (18) are genes which encode the keto group-introducing enzymes (referred to hereinafter as crtW). Typical examples of the genes are crtW genes cloned from the marine bacteria Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes sp. PC-1, which are the DNA strands comprising the nucleotide sequences encoding the polypeptides having the amino acid sequences A - B in Fig. 1 (amino acid Nos. 1 - 212 in SEQ ID NOS: 1) or A - B in Figs. 13 - 14 (amino acid Nos. 1 - 242 in SEQ ID NOS: 5). The crtW gene product (also referred to hereinafter as CrtW) has an enzyme activity for converting the 4-methylene group of the β -ionone ring into a keto group, and one of the specific examples is an enzyme activity for synthesizing canthaxanthin with β -carotene as a substrate by way of echinenone (see Fig. 11). In addition, the crtW gene product also has an enzyme activity for converting the 4-methylene group of the 3-hydroxy- β -ionone ring into a keto group, and one of the specific examples is an enzyme activity for synthesizing astaxanthin with zeaxanthin as a substrate by way of 4-ketozeaxanthin (see Fig. 11). In this connection, the polypeptides having such enzyme activities and the DNA strands encoding the polypeptides have not hitherto been reported, and the polypeptides or the DNA strands encoding the polypeptides has no overall homology to polypeptides or DNA strands which have hitherto been reported. Moreover, no such information has been reported that one enzyme has an activity to convert directly a dihydrocarbonyl group of not only the β -ionone ring and the 3-hydroxy- β -ionone ring but also the other compounds into a keto group. Moreover, a homology of CrtW as high as 83% identity at an amino acid sequence level was shown between Agrobacterium and Alcaligenes.

On the other hand, it is possible to allow a microorganisms such as Escherichia coli or the like to produce β -carotene or zeaxanthin by using the carotenoid synthesis genes of the non-photosynthetic bacterium Erwinia, that is the crtE, crtB, crtI and crtY genes of Erwinia afford the microorganism such as Escherichia coli or the like the β -carotene-producing ability, and the crtE, crtB, crtI, crtY and crtZ genes of Erwinia afford the microorganisms such as Escherichia coli or the like the zeaxanthin-producing ability (see Fig. 10 and Laid-Open Publication of WO91/13078). Thus, the substrate of CrtW is supplied by the crt gene cluster of Erwinia, so that when additional crtW gene is introduced into the microorganism such as Escherichia coli or the like which contains the aforementioned crt gene cluster of Erwinia, the β -carotene-producing microorganism will produce canthaxanthin by way of echinenone, and the zeaxanthin-producing microorganism will produce astaxanthin by way of 4-ketozeaxanthin.

Hydroxyl group-introducing enzyme gene (crtZ)

The DNA strands (19) - (27) are genes encoding a hydroxyl group-introducing enzyme (referred to hereinafter as crtZ). Typical examples of the genes are crtZ genes cloned from the marine bacteria Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes sp. PC-1, which are the DNA strands comprising the nucleotide sequences encoding the polypeptides having the amino acid sequences C - D in Fig. 2 (amino acid Nos. 1 - 162 in SEQ ID NOS: 2) or C - D in Figs. 15 (amino acid Nos. 1 - 162 in SEQ ID NOS: 6). The crtZ gene product (also referred to hereinafter as CrtZ) has an enzyme activity for adding a hydroxyl group to the 3-carbon atom of the β -ionone ring, and one of the specific examples is an enzyme activity for synthesizing zeaxanthin with use of β -carotene as a substrate by way of β -cryptoxanthin (see Fig. 11). In addition, the crtZ gene product also has an enzyme activity for adding a hydroxyl group to the 3-carbon atom of the 4-keto- β -ionone ring, and one of the specific examples is an enzyme activity for synthesizing astaxanthin

with canthaxanthin as a substrate by way of phoenicoxanthin (see Fig. 11). In this connection, the polypeptide having the latter enzyme activity and the DNA strand encoding the polypeptide have not hitherto been reported. Moreover, CrtZ of *Agrobacterium* and *Alcaligenes* showed a high homology with CrtZ of *Erwinia uredovora* (57% and 58% identity), respectively, at an amino acid sequence level. Also, a high homology of 90% identity at an amino acid sequence level was shown between the CrtZ of *Agrobacterium* and *Alcaligenes*.

It has been described above that it is possible to allow a microorganism such as *Escherichia coli* or the like to produce β -carotene by using the carotenoid synthetic genes of the non-photosynthetic bacterium *Erwinia*. Moreover, it has been described above that it is possible to allow a microorganism such as *Escherichia coli* or the like to produce canthaxanthin by adding crtW thereto. Thus, the substrate of CrtZ of *Agrobacterium* or *Alcaligenes* is supplied by the crtE, crtB, crtI and crtY genes of *Erwinia* (production of β -carotene), and the crtW gene of *Agrobacterium* or *Alcaligenes* added thereto, so that when the crtZ gene of *Agrobacterium* or *Alcaligenes* is introduced into a microorganism such as *Escherichia coli* or the like containing the crt gene group, the β -carotene-producing microorganism will produce zeaxanthin by way of β -cryptoxanthin, and the canthaxanthin-producing microorganism will produce astaxanthin by way of phoenicoxanthin.

Lycopene-cyclizing enzyme gene (crtY)

The DNA strand encoding the amino acid sequence substantially from E to F of Figs. 3 and 4 (amino acid Nos. 1-386 in SEQ ID NO: 3) is a gene encoding a lycopene-cyclizing enzyme (referred to hereinafter as crtY). A typical example of the gene is the crtY gene cloned from the marine bacterium *Agrobacterium aurantiacus* sp. nov. MK1, which is the DNA strand comprising the nucleotide sequence encoding the polypeptide having the amino acid sequence E - F in Figs. 3 and 4. The crtY gene product (also referred to hereinafter as CrtY) has an enzyme activity for synthesizing β -carotene with lycopene as a substrate (see Fig. 11). It is possible to allow a microorganism such as *Escherichia coli* or the like to produce lycopene by using a carotenoid biosynthesis genes of a non-photosynthetic bacterium *Erwinia*, that is the crtE, crtB and crtI genes of *Erwinia* give a microorganism such as *Escherichia coli* or the like a lycopene biosynthesis ability (see Fig. 10, and Laid-Open Publication of WO91/13078). Thus, the substrate of the CrtY of *Agrobacterium* is supplied by the crt gene group of *Erwinia*, so that when the crtY of *Agrobacterium* is introduced into a microorganism such as *Escherichia coli* or the like containing the crt gene group, it is possible to allow the microorganism to produce β -carotene.

In this connection, the CrtY of *Agrobacterium* has a significant homology of 44.3% identity to the CrtY of *Erwinia uredovora* at the amino acid sequence level, and these CrtY enzymes also have the same enzymatic function (see Figs. 10 and 11).

Bacteriological properties of marine bacteria

The marine bacteria *Agrobacterium aurantiacus* sp. nov. MK1 and *Alcaligenes* sp. PC-1 as the sources of the xanthophyll synthetic genes show the following bacteriological properties.

Agrobacterium aurantiacus sp. nov. MK1)

(1) Morphology

Form and size of bacterium: rod, $0.9 \mu\text{m} \times 1.2 \mu\text{m}$;

Motility: yes;

Flagellum: peripheric flagellum;

Polymorphism of cell: none;

Sporogenesis: none;

Gram staining: negative.

(2) Growths in culture media

Broth agar plate culture: non-diffusive circular orange colonies having a gloss are formed.

Broth agar slant culture: a non-diffusive orange band having a gloss is formed.

Broth liquid culture: homogeneous growth all over the culture medium with a color in orange.

Broth gelatin stab culture: growth over the surface around the stab pore.

(3) Physiological properties

Reduction of nitrate: positive;

Denitrification reaction: negative;

Formation of indole: negative;

Utilization of citric acid: negative;

Formation of pigments: fat-soluble reddish orange pigment;

Urease activity: negative;

Oxidase activity: positive;

Catalase activity: positive;

5 β -Glucosidase activity (esculin degradability): positive;

β -Galactosidase activity: positive;

Growth range: pH, 5 - 9; temperature, 10 - 40°C;

Behavior towards oxygen: aerobic;

Durability to seawater: positive;

10 O - F test: oxidation;

Anabolic ability of saccharides:

Positive: D-glucose, D-mannose, D-galactose, D-fructose, lactose, maltose, sucrose, glycogen, N-acetyl-D-glucosamine;

Negative: L-arabinose, D-mannitol, inositol, L-rhamnose, D-sorbitol;

15 Anabolic ability of organic acids:

Positive: lactate;

Negative: citrate, malate, gluconate, caprylate, succinate, adipate;

Anabolic ability of the other organic materials:

Positive: inosine, uridine, glucose-1-phosphate, glucose-6-phosphate;

20 Negative: gelatin, L-arginine, DNA, casein.

(Alcaligenes sp. PC-1)

(1) Morphology

25 Form and size of bacterium: short rod, 1.4 μ m;

Motility: yes;

Flagellum: peripheric flagellum;

Polymorphism of cell: none;

Sporogenesis: none;

30 Gram staining: negative.

(2) Growths in culture media

Broth agar plate culture: non-diffusive circular orange colonies having a gloss are formed.

Broth agar slant culture: a non-diffusive orange band having a gloss is formed.

35 Broth liquid culture: homogeneous growth all over the culture medium with a color in orange.

Broth gelatin stab culture: growth over the surface around the stab pore.

(3) Physiological properties:

Formation of pigments: fat-soluble reddish orange pigment;

40 Oxidase activity: positive;

Catalase activity: positive;

Growth range: pH, 5 - 9; temperature, 10 - 40°C;

Behavior towards oxygen: aerobic;

Durability to seawater: positive;

45 O - F test: oxidation;

Degradability of gelatin: negative.

Xanthophyll synthetic gene cluster of the other marine bacteria

50 It has hitherto been reported that 16 marine bacteria have an ability to synthesize ketocarotenoids such as astaxanthin and the like (Yokoyama, A., Izumida, H., Miki, W., "Marine bacteria produced astaxanthin", 10th International Symposium on Carotenoids, Abstract, CL11-3, 1993). If either of the crt genes of the aforementioned marine bacteria Agrobacterium aurantiacus sp. nov. MK-1 or Alcaligenes sp. PC-1 is used as a probe, the gene cluster playing a role of the biosynthesis of ketocarotenoids such as astaxanthin and the like should be obtained from the other astaxanthin producing marine bacteria by using the homology of the genes. In fact, the present inventors have successfully obtained the crtW and crtZ genes as the strongly hybridizing DNA fragments from the chromosomal DNA of Alcaligenes PC-1 with use of a DNA fragment containing crtW and crtZ of Ag. aurantiacus sp. nov. MK1 as a probe (see Examples as for the details). Furthermore, when Alteromonas SD-402 was selected from the remaining 14 marine bacteria having an astaxanthin synthetic ability and a chromosomal DNA was prepared therewith and subjected to the Southern hybridi-

zation experiment with a DNA fragment containing *crtW* and *crtZ* of *Ag. aurantiacus* sp. nov. MK1, the probe hybridized with the bands derived from the chromosomal DNA of the marine bacteria. The DNA strands according to the present invention also includes a DNA strand which hybridizes with the DNA strands (2), (4), (6), (8), (11), (13), (15), (17), (20), (22), (24) and (26).

Acquisition of DNA strands

Although one of the methods for obtaining the DNA strand having a nucleotide sequence which encodes the amino acid sequence of each enzyme described above is to chemically synthesize at least a part of the strand length according to the method for synthesizing a nucleic acid, it is believed more preferable than the chemical synthetic method to obtain the DNA strand by using the total DNA having been digested with an appropriate restriction enzyme to prepare a library in *Escherichia coli*, from which library the DNA strand is obtained by the methods conventionally used in the art of genetic engineering such as a hybridization method with an appropriate probe (see the xanthophyll synthetic gene cluster of the other marine bacteria).

Transformation of an microorganism such as *Escherichia coli* and gene expression

A variety of xanthophylls can be prepared by introducing the present DNA strands described above into appropriate microorganisms such as bacteria, for example *Escherichia coli*, *Zymomonas mobilis* and *Agrobacterium tumefaciens*, and yeasts, for example *Saccharomyces cerevisiae*.

The outline for introducing an foreign gene into a preferred microorganism is described below.

The procedure or method for introducing and expressing the foreign gene in a microorganism such as *Escherichia coli* or the like comprises the ones usually used in the art of genetic engineering in addition to those described below in the present invention and may be carried out according to the procedure or method (see, e.g., "Vectors for Cloning Genes", Methods in Enzymology, 216, p. 469-631, 1992, Academic Press, and "Other Bacterial Systems", Methods in Enzymology, 204, p. 305-636, 1991, Academic Press).

(*Escherichia coli*)

The method for introducing foreign genes into *Escherichia coli* includes several efficient methods such as the Hana-han's method and the rubidium method, and the foreign genes may be introduced according to these methods (see, for example, Sambrook, J., Fritsch, E.F., Maniatis, T., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989). While foreign genes in *Escherichia coli* may be expressed according to the conventional methods (see, for example, "Molecular Cloning - A Laboratory Manual"), the expression can be carried out for example with a vector for *Escherichia coli* having a lac promoter in the pUC or pBluescript series. The present inventors have used a vector pBluescrip II SK or KS for *Escherichia coli* having a lac promoter and the like to insert the *crtW*, *crtZ* and *crtY* genes of *Agrobacterium aurantiacus* sp. nov. MK1 and the *crtW* and *crtZ* genes of *Alcaligenes* sp. PC-1 and allowed to express these genes in *Escherichia coli*.

(Yeast)

The method for introducing foreign genes into yeast *Saccharomyces cerevisiae* includes the methods which have already been established such as the lithium method and the like, and the introduction may be carried out according to these methods (see, for example, Ed. Yuichi Akiyama, compiled by Bio-industry Association, "New Biotechnology of Yeast", published by IGAKU SHUPPAN CENTER). Foreign genes can be expressed in yeast by using a promoter and a terminator such as PGK and GPD to construct an expression cassette in which the foreign gene is inserted between the promoter and the terminator so that transcription is led through, and inserting the expression cassette into a vector such as the YRp system which is a multi-copy vector for yeast having the ARS sequence of the yeast chromosome as the replication origin, the YEp system which is a multi-copy vector for yeast having the replication origin of the 2 μ m DNA of yeast, and the YIp system which is a vector for integrating a yeast chromosome having no replication origin of yeast (see "New Biotechnology of Yeast", published by IGAKU SHUPPAN CENTER, *ibid.*; NIPPON NOGEI-KAGAKU KAI ABC Series "Genetic Engineering for Producing Materials", published by ASAKURA SHOTEN; and Yamano, S., Ishii, T., Nakagawa, M., Ikenaga, H., Misawa, N., "Metabolic Engineering for Production of β -carotene and lycopene in *Saccharomyces cerevisiae*", Biosci. Biotech. Biochem., 58, p. 1112-1114, 1994).

(*Zymomonas mobilis*)

Foreign genes can be introduced into an ethanol-producing bacterium *Zymomonas mobilis* by the conjugal transfer method which is common to Gram-negative bacteria, and the foreign genes can be expressed by using a vector pZA22

for Zymomonas mobilis (see Katsumi Nakamura, "Molecular Breeding of Zymomonas mobilis", Nippon Nogei-Kagaku Kaishi, 63, p. 1016-1018, 1989; and Misawa, N., Yamano, S., Ikenaga, H., "Production of β -Carotene in Zymomonas mobilis and Agrobacterium tumefaciens by Introduction of the Biosynthesis Genes from Erwinia uredovora", Appl. Environ. Microbiol., 57, p.1847-1849, 1991).

(Agrobacterium tumefaciens)

Foreign genes can be introduced into a plant pathogenic bacterium Agrobacterium tumefaciens by the conjugal transfer method which is common to Gram-negative bacteria, and the foreign genes can be expressed by using a vector pBI121 for a bacterium such as Agrobacterium tumefaciens (see Misawa, N., Yamano, S., Ikenaga, H., "Production of β -Carotene in Zymomonas mobilis and Agrobacterium tumefaciens by Introduction of the Biosynthesis Genes from Erwinia uredovora", Appl. Environ. Microbiol., 57, p. 1847-1849, 1991).

Production of xanthophylls by microorganisms

The gene cluster for the synthesis of ketocarotenoids such as astaxanthin derived from a marine bacterium can be introduced and expressed by the procedure or method described above for introducing and expressing a foreign gene in a microorganism.

Farnesyl pyrophosphate (FPP) is a substrate which is common not only to carotenoids but also to other terpenoids such as sesquiterpenes, triterpenes, sterols, hopanols and the like. In general, microorganisms synthesize terpenoids even if they cannot synthesize carotenoids, so that all of the microorganisms should basically have FPP as an intermediate metabolite. Furthermore, the carotenoid synthesis gene cluster of a non-photosynthetic bacterium Erwinia has an ability to synthesize the substrates of the crt gene products of Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes sp. PC-1 by using FPP as a substrate (see Fig. 10). The present inventors have already confirmed that when the group of crt genes of Erwinia is introduced into not only Escherichia coli but also the aforementioned microorganisms, that is the yeast Saccharomyces cerevisiae, the ethanol producing bacterium Zymomonas mobilis, or the plant pathogenic bacterium Agrobacterium tumefaciens, carotenoids such as β -carotene and the like can be produced, as was expected, by these microorganisms (Yamano, S., Ishii, T., Nakagawa, M., Ikenaga, H., Misawa, N., "Metabolic Engineering for Production of β -Carotene and Lycopene in Saccharomyces cerevisiae", Biosci. Biotech. Biochem., 58, p. 1112-1114, 1994; Misawa, N., Yamano, S., Ikenaga, H., "Production of β -Carotene in Zymomonas mobilis and Agrobacterium tumefaciens by Introduction of the Biosynthetic Genes from Erwinia uredovora", Appl. Environ. Microbiol., 57, p. 1847-1849, 1991; and Japanese Patent Application No. 58786/1991 (Japanese Patent Application No. 53255/1990) by the present inventors: "DNA Strands useful for the Synthesis of Carotenoids").

Thus, it should be possible in principle to allow all of the microorganisms, in which the gene introduction and expression system has been established, to produce ketocarotenoids such as astaxanthin and the like by introducing the combination of the carotenoid synthesis gene cluster derived from Erwinia and the DNA strands according to the present invention (typically the carotenoid synthesis gene cluster derived from Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes sp. PC-1) at the same time into the same microorganism. The process for producing a variety of ketocarotenoids in microorganisms are described below.

(Production of canthaxanthin and echinenone)

It is possible to produce canthaxanthin as a final product and echinenone as an intermediate metabolite by introducing into a microorganism such as Escherichia coli and expressing the crtE, crtB, crtI and crtY genes of Erwinia uredovora required for the synthesis of β -carotene and any one of the DNA strands of the present invention (1) - (9) which is a keto group-introducing enzyme gene (typically, the crtW gene of Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes PC-1). The yields or the ratio of canthaxanthin and echinenone can be changed by controlling the expression level of the DNA strand (crtW gene) or examining the culturing conditions of a microorganism having the DNA strand. Two embodiments in Escherichia coli are described below, and more details will be illustrated in Examples.

A plasmid pACCAR16 Δ crtX that a fragment containing the crtE, crtB, crtI and crtY genes of Erwinia uredovora has been inserted into the Escherichia coli vector pACYC184 and a plasmid pAK916 that a fragment containing the crtW gene of Agrobacterium aurantiacus sp. nov. MK1 has been inserted into the Escherichia coli vector pBluescript II SK- were introduced into Escherichia coli JM101 and cultured to the stationary phase to collect bacterial cells and to extract carotenoid pigments. The extracted pigments comprised 94% of canthaxanthin and 6% of echinenone. Also, canthaxanthin was obtained in a yield of 3 mg starting from 2 liters of the culture solution.

A plasmid pACCAR16 Δ crtX that a fragment containing the crtE, crtB, crtI and crtY genes of Erwinia uredovora has been inserted into the Escherichia coli vector pACYC184 and a plasmid pPC17-3 that a fragment containing the crtW gene of Alcaligenes PC-1 has been inserted into the Escherichia coli vector pBluescript II SK+ were introduced into Escherichia coli JM101 and cultured to the stationary phase to collect bacterial cells and to extract carotenoid pigments.

The extracted pigments comprised 40% of canthaxanthin and 50% of echinenone. The remainder comprised 10% of unreacted β -carotene.

(Production of astaxanthin and 4-ketozeaxanthin)

It is possible to produce astaxanthin as a final product and 4-ketozeaxanthin as an intermediate metabolite by introducing into a microorganism such as Escherichia coli or the like and expressing the crtE, crtB, crtI, crtY and crtZ genes of Erwinia uredovora required for the synthesis of zeaxanthin and any one of the DNA strands of the present invention (10) - (18) which is a keto group-introducing enzyme gene (typically, the crtW gene of Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes PC-1). The yields or the ratio of astaxanthin and 4-ketozeaxanthin can be changed by controlling the expression level of the DNA strand (crtW gene) or examining the culturing conditions of a microorganism having the DNA strand.

Two embodiments in Escherichia coli are described below, and more details will be illustrated in Examples.

A plasmid pACCAR25 Δ crtX that a fragment containing the crtE, crtB, crtI, crtY and crtZ genes of Erwinia uredovora has been inserted into the Escherichia coli vector pACYC184 and a plasmid pAK916 that a fragment containing the crtW gene of Ag. aurantiacus sp. nov. MK1 has been inserted into the Escherichia coli vector pBluescript II SK- were introduced into Escherichia coli JM101 and cultured to the stationary phase to collect bacterial cells and to extract carotenoid pigments. The yield of the extracted pigments was 1.7 mg of astaxanthin and 1.5 mg of 4-ketozeaxanthin based on 2 liters of the culture solution.

A plasmid pACCAR25 Δ crtX that a fragment containing the crtE, crtB, crtI, crtY and crtZ genes of Erwinia uredovora has been inserted into the Escherichia coli vector pACYC184 and a plasmid pPC17-3 that a fragment containing the crtW gene of Alcaligenes PC-1 has been inserted into the Escherichia coli vector pBluescript II SK+ were introduced into Escherichia coli JM101 and cultured to the stationary phase to collect bacterial cells and to extract carotenoid pigments. The yield of the extracted pigments was about 1 mg of astaxanthin and 4-ketozeaxanthin, respectively based on 2 liters of the culture solution.

(Production of astaxanthin and phoenicoxanthin)

It is possible to produce astaxanthin as a final product and phoenicoxanthin as an intermediate metabolite by introducing into a microorganism such as Escherichia coli or the like and expressing the crtE, crtB, crtI and crtY genes of Erwinia uredovora required for the synthesis of β -carotene, any one of the DNA strands of the present invention (1) - (9) which is a keto group-introducing enzyme gene (typically, the crtW gene of Agrobacterium aurantiacus sp. nov. MK1 or Alcaligenes PC-1), and any one of the DNA strands of the present invention (19) - (27) which is a hydroxyl group-introducing enzyme gene (typically, the crtZ gene of Ag. aurantiacus sp. nov. MK1 or Alcaligenes PC-1). The yields or the ratio of astaxanthin and phoenicoxanthin can be changed by controlling the expression level of the DNA strands (crtW and crtZ genes) or examining the culturing conditions of a microorganism having the DNA strands. An embodiment in Escherichia coli are described below, and more details will be illustrated in Examples.

A plasmid pACCAR16 Δ crtX that a fragment containing the crtE, crtB, crtI and crtY genes of Erwinia uredovora has been inserted into the Escherichia coli vector pACYC184 and a plasmid pAK96K that a fragment containing the crtW and crtZ genes of Ag. aurantiacus sp. nov. MK1 has been inserted into the Escherichia coli vector pBluescript II SK- were introduced into Escherichia coli JM101 and cultured to the stationary phase to collect bacterial cells and to extract carotenoid pigments. The yield of the extracted pigments comprised was 3 mg of astaxanthin and 2 mg of phoenicoxanthin starting from 4 liters of the culture solution.

Deposition of microorganisms

Microorganisms as the gene sources of the DNA strands of the present invention and Escherichia coli carrying the isolated genes (the DNA strands of the present invention) have been deposited to National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology.

(i) Agrobacterium aurantiacus sp. nov. MK1

Deposition No: FERM BP-4506

Entrusted Date: December 20, 1993

(ii) Escherichia coli JM101 (pAccrt-EIB, pAK92)

Deposition No: FERM BP-4505

Entrusted Date: December 20, 1993

(iii) Alcaligenes sp. PC-1

Deposition No: FERM BP-4760

Entrusted Date: July 27, 1994

(iv) Escherichia coli β : pPC17
Deposition No: FERM BP-4761
Entrusted Date: July 27, 1994

5 Examples

The present invention is further described more specifically with reference to the following examples without restriction of the invention. In addition, the ordinary experiments of gene manipulation employed herein is based on the standard methods (Sambrook, J., Fritsch, E.F., Maniatis, T., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989), unless otherwise specified.

Example 1: Preparation of chromosomal DNA

Chromosomal DNAs were prepared from three marine bacterial strains, i.e. Agrobacterium aurantiacus sp. nov. MK1, Alcaligenes sp. PC-1, and Alteromonas SD-402 (Yokoyama, A., Izumida, H., Miki, W., "Marine bacteria produced astaxanthin", 10th International Symposium on Carotenoids, Abstract, CL11-3, 1993). After each of these marine bacteria was grown in 200 ml of a culture medium (a culture medium prepared according to the instruction of "Marine Broth" manufactured by DIFCO) at 25°C for 4 days to the stationary phase, the bacterial cells were collected, washed with a TES buffer (20 mM Tris, 10 mM EDTA, 0.1 M NaCl, pH 8), subjected to heat treatment at 68°C for 15 minutes, and suspended into the solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8) containing 5 mg/ml of lysozyme (manufactured by SEIKAGAKU KOGYO) and 100 μ g/ml of RNase A (manufactured by Sigma). After incubation of the suspension at 37°C for 1 hour, Proteinase K (manufactured by Boehringer-Mannheim) was added and the mixture was incubated at 37°C for 10 minutes. After SARCOSIL (N-lauroylsarcosine Na, manufactured by Sigma) was then added at the final concentration of 1% and the mixture was sufficiently mixed, it was incubated at 37°C for several hours. The mixture was extracted several times with phenol/chloroform, and ethanol in a two-time amount was added slowly. Chromosomal DNA thus deposited was wound around a glass rod, rinsed with 70% ethanol and dissolved in 2 ml of a TE buffer (10 mM Tris, 1 mM EDTA, pH 8) to prepare a chromosomal DNA solution.

Example 2: Preparation of hosts for a cosmid library

(1) Preparation of phytoene-producing Escherichia coli

After the removal of the BstEII (1235) - Eco521 (4926) fragment from a plasmid pCAR16 having a carotenoid synthesis gene cluster except the crtZ gene of Erwinia uredovora (Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K., Harashima, K., "Elucidation of the Erwinia uredovora Carotenoid Biosynthetic Pathway by Functional Analysis of Gene Products expressed in Escherichia coli", J. Bacteriol., 172, p. 6704-6712, 1990; and Japanese Patent Application No. 58786/1991 (Japanese Patent Application No. 53255/1990): "DNA Strands useful for the Synthesis of Carotenoids"), a 2.3 kb Asp718 (KpnI) - EcoRI fragment containing the crtE and crtB genes required for the production of phytoenes was cut out. This fragment was then inserted into the EcoRV site of the E. coli vector pACYC184 to give an aimed plasmid (pACCRT-EB). The bacterium E. coli containing pACCRT-EB exhibits resistance to an antibiotic chloramphenicol (Cm^r) and produces phytoenes (Linden, H., Misawa, N., Chamovitz, D., Pecker, I., Hirschberg, J., Sandmann, G., "Functional Complementation in Escherichia coli of Different Phytoene Desaturase Genes and Analysis of Accumulated Carotenes", Z. Naturforsch., 46c, 1045-1051, 1991).

(2) Preparation of lycopene-producing Escherichia coli

After the removal of the BstEII (1235) - SnaBI (3497) fragment from a plasmid pCAR16 having a carotenoid synthesis gene cluster except the crtZ gene of Erwinia uredovora, a 3.75 kb Asp718 (KpnI) - EcoRI fragment containing the crtE, crtI and crtB genes required for the production of lycopene was cut out. This fragment was then inserted into the EcoRV site of the E. coli vector pACYC184 to give an aimed plasmid (pACCRT-EIB). The bacterium E. coli containing pACCRT-EIB exhibits Cm^r and produces lycopene (Cunningham Jr, F.X., Chamovitz, D., Misawa, N., Gatt, E., Hirschberg, J., "Cloning and Functional Expression in Escherichia coli of Cyanobacterial Gene for Lycopene Cyclase, the Enzyme that catalyzes the Biosynthesis of β -Carotenes", FEBS Lett., 328, 130-138, 1993).

(3) Preparation of β -carotene-producing Escherichia coli

After the crtX gene was inactivated by subjecting a plasmid pCAR16 having a carotenoid synthesis gene cluster except the crtZ gene of Erwinia uredovora to digestion with restriction enzyme BstEII, the Klenow fragment treatment and the ligation reaction, a 6.0 kb Asp718 (KpnI) - EcoRI fragment containing crtE, crtY, crtI and crtB genes required

for the production of β -carotene was cut out. This fragment was then inserted into the *EcoRV* site of the *E. coli* vector pACYC184 to give an aimed plasmid (referred to hereinafter as pACCAR16 Δ crtX). The bacterium *E. coli* containing pACCAR16 Δ crtX exhibits Cm^r and produces β -carotene. In this connection, the restriction enzyme and enzymes used for genetic manipulation have been purchased from TAKARA SHUZO (K.K.) or Boehringer-Mannheim.

Example 3: Preparation of a cosmid library and acquisition of *Escherichia coli* which exhibits orange in color

After the restriction enzyme *Sau3AI* was added in an amount of one unit to 25 μ g of the chromosomal DNA of *Agrobacterium aurantiacus* sp. nov. MK1, the mixture was incubated at 37°C for 15 minutes and heat treated at 68°C for 10 minutes to inactivate the restriction enzyme. Under the condition, many partially digested fragments with *Sau3AI* were obtained at about 40 kb. The cosmid vector pJBB (resistant to ampicillin (Ap^r)) which had been subjected to *Bam*HI digestion and alkaline phosphatase treatment and the right arm (shorter fragment) of pJBB which had been digested with *Sall*/*Bam*HI and then recovered from the gel were mixed with a part of the above *Sau3AI* partial fragments, and ligated at 12°C overnight. In this connection, pJBB has been purchased from Amersham.

Phage particles were obtained in an amount sufficient for preparing a cosmid library by the *in vitro* packaging with a Gigapack Gold (manufactured by Stratagene; available from Funakoshi) using the DNA above ligated.

After *Escherichia coli* DH1 (ATCC33849) and *Escherichia coli* DH1, each of which has one of the three plasmids prepared in Example 2, were infected with the phage particles, these bacteria were diluted so that 100 - 300 colonies were found on a plate, plated on LB containing appropriate antibiotics (1% trypton, 0.5% yeast extract, 1% NaCl), and cultured at 37°C or room temperature for a period of overnight to several days.

As a result, in cosmid libraries having the simple *Escherichia coli* (beige) or the phytoene-producing *Escherichia coli* (beige) with pACCRT-EB as a host, no colonies with changed color were obtained notwithstanding the screening of a ten thousand or more of the colonies for respective libraries. On the other hand, in cosmid libraries having the lycopene-producing *Escherichia coli* (light red) with pACCRT-EIB or the β -carotene-producing *Escherichia coli* (yellow) with pACCAR16 Δ crtX as a host, colonies exhibiting orange have appeared in a proportion of one strain to several hundred colonies, respectively. Most of these transformed *Escherichia coli* strains which exhibits orange contained plasmid pJB8 in which about 40 kb partially digested *Sau3AI* fragments were cloned. It is also understood from the fact that no colonies with changed color appeared in cosmid libraries having the simple *Escherichia coli* or the phytoene-producing *Escherichia coli* with pACCRT-EB as a host, that *Escherichia coli* having an ability of producing a carotenoid synthetic intermediate of the later steps of at least phytoene should be used as a host for the purpose of expression-cloning the xanthophyll synthesis gene cluster from the chromosomal DNA of *Agrobacterium aurantiacus* sp. nov. MK1.

Example 4: Localization of a fragment containing an orange pigment synthesis gene cluster

When individual several ten colonies out of the orange colonies obtained in cosmid libraries having the lycopene-producing *Escherichia coli* (light red) with pACCRT-EIB or the β -carotene-producing *Escherichia coli* (yellow) with pACCAR16 Δ crtX as a host were selected to analyze the plasmids, 33 kb - 47 kb fragments partially digested with *Sau3AI* were inserted in vector pJB8 in all of the colonies except one strain. The remaining one strain (lycopene-producing *Escherichia coli* as a host) contains a plasmid, in which a 3.9 kb fragment partially digested with *Sau3AI* was inserted in pJB8 (referred to hereinafter as plasmid pAK9). This was considered to be the one formed by the *in vivo* deletion of the inserted fragment after the infection to *Escherichia coli*. The same pigment (identified as astaxanthin in Example 6) as that in the orange colonies obtained from the other cosmid libraries was successfully synthesized with the lycopene-producing *Escherichia coli* having pAK9, pAK9 was used as a material in the following analyses.

Example 5: Determination of the nucleotide sequence in the orange pigment synthesis gene cluster

A 3.9 kb *EcoRI* inserted fragment prepared from pAK9 was inserted into the *EcoRI* site of the *Escherichia coli* vector pBluescrip II SK+ to give two plasmids (pAK91 and pAK92) with the opposite directions of the fragment to the vector. The restriction enzyme map of one of the plasmids (pAK92) is illustrated in Fig. 12. When pAK92 was introduced into the lycopene-producing *Escherichia coli*, orange colonies were obtained as a result of the synthesis of astaxanthin (Example 6). However, no ability for synthesizing new pigments was afforded even if pAK91 was introduced into the lycopene-producing *Escherichia coli*. It was thus considered that the pigment synthesis gene cluster in the plasmid pAK92 has the same direction as that of the *lac* promoter of the vector. Next, each of a 2.7 kb *Pst*I fragment obtained by the *Pst*I digestion of pAK91, a 2.9 kb *Bam*HI fragment obtained by the *Bam*HI digestion of pAK92, and 2.3 kb and 1.6 kb *Sall* fragments obtained by the *Sall* digestion of pAK92 was cloned into the vector pBluescrip II SK-. The restriction maps of plasmids referred to as pAK94, pAK96, pAK98, pAK910, pAK93, and pAK95 are illustrated in Fig. 12. The plasmids pAK94, pAK96, pAK98 and pAK910 have the pigment synthesis gene cluster in the same direction as that of the *lac* promoter of the vector, while the plasmids pAK93 and pAK95 have the pigment synthesis gene cluster in the opposite direction to that of the promoter.

It was found that when the plasmid pAK96 having a 2.9 kb BamHI fragment was introduced into the lycopene-producing Escherichia coli, the transformant also synthesized astaxanthin as in the case when the plasmid pAK92 having a 3.9 kb EcoRI fragment was introduced (Example 6), so that the DNA sequence of the 2.9 kb BamHI fragment was determined.

The DNA sequence was determined by preparing deletion mutants of the 2.9 kb BamHI fragment from the normal and opposite directions and determining the sequence using clones having various lengths of deletions. The deletion mutants were prepared from the four plasmids pAK96, pAK98, pAK93 and pAK95 according to the following procedure: Each of the plasmids, 10 µg, was decomposed with SacI and XbaI and extracted with phenol/chloroform to recover DNA by ethanol precipitation. Each of DNA was dissolved in 100 µl of ExoIII buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, pH 8.0). 180 units of ExoIII nuclease was added, and the mixture was maintained at 37°C. A 10 µl portion was sampled at every 1 minute, and two samples were transferred into a tube in which 20 µl of MB buffer (40 mM sodium acetate, 100 mM NaCl, 2 mM ZnCl₂, 10% glycerol, pH 4.5) is contained and which is placed on ice. After completion of the sampling, five tubes thus obtained were maintained at 65°C for 10 minutes to inactivate the enzyme, five units of mung bean nuclease were added, and the mixtures were maintained at 37°C for 30 minutes. After the reaction, five DNA fragments different from each other in the degrees of deletion were recovered for each plasmid by agarose gel electrophoresis. The DNA fragments thus recovered was blunt ended with the Klenow fragment, subjected to the ligation reaction at 16°C overnight, and Escherichia coli JM109 was transformed. A single stranded DNA was prepared from each of various clones thus obtained with a helper phage M13K07, and subjected to the sequence reaction with a fluorescent primer cycle-sequence kit available from Applied Biosystem (K.K.), and the DNA sequence was determined with an automatic sequencer.

The DNA sequence comprising 2886 base pairs (bp) thus obtained is illustrated in Figs. 5 - 9 (SEQ ID NO: 4). As a result of examining an open reading frame having a ribosome binding site in front of the initiation codon, three open reading frames which can encode the corresponding proteins (A - B (nucleotide positions 229 - 864 of SEQ ID NO: 4), C - D (nucleotide positions 864 - 1349), E - F (nucleotide positions 1349 - 2506) in Figs. 5 - 9) were found at the positions where the three xanthophyll synthesis genes crtW, crtZ and crtY are expected to be present. For the two open reading frames of A - B and E - F, the initiating codon is GTG, and for the remaining open reading frame C - D, it is ATG.

Example 6: Identification of the orange pigment

The lycopene-producing Escherichia coli JM101 having pAK92 or pAK96 introduced therein (Escherichia coli (pACCRT-EIB, pAK92 or pAK96); exhibiting orange) or the β-carotene-producing Escherichia coli JM101 having pAK94 or pAK96K (Fig. 12) introduced therein (Escherichia coli (pACCAR16ΔcrtX, pAK94 or pAK96K); exhibiting orange) was cultured in 4 liters of a 2YT culture medium (1.6% trypton, 1% yeast extract, 0.5% NaCl) containing 150 µg/ml of ampicillin (Ap, manufactured by Meiji Seika) and 30 µg/ml of chloramphenicol (Cm, manufactured by Sankyo) at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 600 ml of acetone, concentrated, extracted twice with 400 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (15/1). The original orange pigment was separated into three spots at the R_f values of 0.72, 0.82 and 0.91 by TLC. The pigment of the darkest spot at R_f 0.72 corresponding to 50% of the total amount of orange pigment and the pigment of secondly darker spot at R_f 0.82 were scratched off from the TLC plate, dissolved in a small amount of chloroform/methanol (9/1) or methanol, and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or methanol to give purified materials in a yield of 3 mg (R_f 0.72) and 2 mg (R_f 0.82), respectively.

It has been elucidated from the results of the UV-visible, ¹H-NMR and FD-MS (m/e 596) spectra that the pigment at R_f 0.72 has the same planar structure as that of astaxanthin. When the pigment was dissolved in diethyl ether : 2-propanol : ethanol (5 : 5 : 2) to measure the CD spectrum, it was proved to have stereochemical configuration of 3S, 3'S, and thus identified as astaxanthin; see Fig. 11 for the structural formula). Also, the pigment at R_f 0.82 was identified as phoenicoxanthin (see Fig. 11 for the structural formula) from the results of its UV-visible, ¹H-NMR and FD-MS (m/e 580) spectra. In addition, the pigment at 0.91 was canthaxanthin (Example 7(2)).

Example 7: Identification of metabolic intermediates of xanthophyll

(1) Identification of 4-ketozeaxanthin

The zeaxanthin producing Escherichia coli was prepared according to the following procedure. That is to say, the plasmid pCAR25 having total carotenoid synthesis gene cluster of Er. uredorora (Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K., Harashima, K., "Elucidation of the Erwinia uredovora Carotenoid Biosynthetic Pathway by Functional Analysis of Gene Products expressed in Escherichia coli", J. Bacteriol., 172, p. 6704-6712, 1990; and Japanese Patent Application No. 58786/1991 (Japanese Patent Application No. 53255/1990): "DNA

Strands useful for the Synthesis of Carotenoids") was digested with restriction enzyme BstEII, and subjected to the Klenow fragment treatment and ligation reaction to inactivate the crtX gene by reading frame shift, and then a 6.5 kb Asp718 (KpnI) - EcoRI fragment containing the crtE, crtY, crtI, crtB and crtZ genes required for producing zeaxanthin was cut out. This fragment was then inserted into the EcoRV site of the Escherichia coli vector pACYC184 to give the aimed plasmid (referred to hereinafter as pACCAR25ΔcrtX).

The zeaxanthin-producing Escherichia coli JM101 having pAK910 or pAK916 (Fig. 12) introduced thereinto (Escherichia coli (pACCAR25ΔcrtX, pAK910 or pAK916); exhibiting orange) was cultured in 2 liters of a 2YT culture medium containing 150 μg/ml of Ap and 30 μg/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, extracted twice with 200 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (15/1). The original orange pigment was separated into three spots at the R_f values of 0.54 (46%), 0.72 (53%) and 0.91 (1%) by TLC. The pigment at R_f 0.54 was scratched off from the TLC plate, dissolved in a small amount of chloroform/methanol (9/1) or methanol, and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or methanol to give a purified material in a yield of 1.5 mg.

This material was identified as 4-ketozeaxanthin (see Fig. 11 for the structural formula) since its UV-visible spectrum, FD-MS spectrum (m/e 582) and mobility in silica gel TLC (developed with chloroform/methanol (15/1)) accorded perfectly with those of the standard sample of 4-ketozeaxanthin (purified from Agrobacterium aurantiacus sp. nov. MK1; Japanese Patent Application No. 70335/1993). In addition, the pigments at R_f 0.72 and 0.91 are astaxanthin (Example 6) and canthaxanthin (Example 7 (2)), respectively.

(2) Identification of canthaxanthin

The β-carotene producing Escherichia coli JM101 having pAK910 or pAK916 introduced thereinto (Escherichia coli (pACCAR16ΔcrtX, pAK910 or pAK916); exhibiting orange) was cultured in 2 liters of a 2YT culture medium containing 150 μg/ml of Ap and 30 μg/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, extracted twice with 200 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (50/1). The pigment of the darkest spot corresponding to 94% of the total amount of orange pigments was scratched off from the TLC plate, dissolved in a small amount of chloroform/methanol (9/1) or chloroform/methanol (1/1), and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or chloroform/methanol (1/1) to give a purified material in a yield of 3 mg.

This material was identified as canthaxanthin (see Fig. 11 for the structural formula) since its UV-visible, ¹H-NMR, FD-MS (m/e 564) spectra and mobility in silica gel TLC (R_f 0.53 on developing with chloroform/methanol (50/1)) accorded perfectly with those of the standard sample of canthaxanthin (manufactured by BASF). In addition, the pigment corresponding to 6% of the total orange pigments found in the initial extract was considered echinenone (see Fig. 11 for the structural formula) on the basis of its UV-visible spectrum, mobility in silica gel TLC (R_f 0.78 on developing with chloroform/methanol (50/1)), and mobility in HPLC with NOVA PACK HR 6μ C18 (3.9 × 300 mm; manufactured by Waters) (RT 16 minutes on developing at a flow rate of 1.0 ml/min with acetonitrile/methanol/2-propanol (90/6/4)).

(3) Identification of zeaxanthin

The β-carotene-producing Escherichia coli JM101 having pAK96NK introduced thereinto (Escherichia coli (pACCAR16ΔcrtX, pAK96NK); exhibiting yellow) was cultured in 2 liters of a 2YT culture medium containing 150 μg/ml of Ap and 30 μg/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, extracted twice with 200 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (9/1). The pigment of the darkest spot corresponding to 87% of the total amount of yellow pigments was scratched off from the TLC plate; dissolved in a small amount of chloroform/methanol (9/1) or methanol, and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or methanol to give a purified material in a yield of 3 mg.

It has been elucidated that this material has the same planar structure as that of zeaxanthin since its UV-visible, ¹H-NMR, FD-MS (m/e 568) spectra and mobility in silica gel TLC (R_f 0.59 on developing with chloroform/methanol (9/1)) accorded perfectly with those of the standard sample of zeaxanthin (manufactured by BASF). When the pigment was dissolved in diethyl ether : 2-propanol : ethanol (5 : 5 : 2) to measure the CD spectrum, it was proved to have a stereochemical configuration of 3R, 3'R, and thus identified as zeaxanthin (see Fig. 11 for the structural formula). Also, the pigment corresponding to 13% of the total yellow pigments found in the initial extract was considered β-cryptoxan-

thin (see Fig. 11 for the structural formula) on the basis of its UV-visible spectrum, mobility in silica gel TLC (Rf 0.80 on developing with chloroform/methanol (9/1)), and mobility in HPLC with NOVA PACK HR 6 μ C18 (3.9 \times 300 mm; manufactured by Waters) (RT 19 minutes on developing at a flow rate of 1.0 ml/min with acetonitrile/methanol/2-propanol (90/6/4)).

(4) Identification of β -carotene

The lycopene-producing *Escherichia coli* JM101 having pAK98 introduced therein (*Escherichia coli* (pACCRT-EIB, pAK98); exhibiting yellow) was cultured in 2 liters of a 2YT culture medium containing 150 μ g/ml of Ap and 30 μ g/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, and extracted twice with 200 ml of hexane. The hexane layer was concentrated and chromatographed on a silica gel column (15 \times 300 mm) with an eluent of hexane/ethyl acetate (50/1) to give 3 mg of a purified material.

The material was identified as β -carotene (see Fig. 11 for the structural formula), since all of the data of its UV-visible, FD-MS spectrum (m/e 536) and mobility in HPLC with NOVA PACK HR 6 μ C18 (3.9 \times 300 mm; manufactured by Waters) (RT 62 minutes on developing at a flow rate of 1.0 ml/min with acetonitrile/methanol/2-propanol (90/6/4)) accorded with those of the standard sample of β -carotene (all trans type; manufactured by Sigma).

Example 8: Identification of xanthophyll synthesis gene cluster

(1) Identification of a keto group-introducing enzyme gene

It is apparent from the results of Example 6 that among the 3.9 kb fragment contained in pAK9 (Example 4) or pAK92, all of the genes required for the synthesis of astaxanthin from lycopene is contained in the 2.9 kb BamHI fragment at the right side (pAK96, Fig. 12). Thus, the 1.0 kb fragment at the left side is not needed. Unique NcoI and KpnI sites are present within the 2.9 kb BamHI fragment of pAK96. It is found from the results of Example 7 (3) that the 1.4 kb fragment (pAK96NK) between the NcoI and KpnI sites has a hydroxyl group-introducing enzyme activity but has no keto group-introducing enzyme activity. Canthaxanthin can also be synthesized from β -carotene with the 2.9 kb BamHI fragment from which a fragment of the right side from unique SalI site between the NcoI and KpnI sites had been removed (pAK910) or with the 2.9 kb BamHI fragment from which a fragment of the right side from the HincII site positioned at the left side of the SalI site had been removed (pAK916), but activity for synthesizing canthaxanthin from β -carotene disappeared in the 2.9 kb BamHI fragment of pAK96 from which a fragment of the right side from the NcoI site left of the HincII site had been removed. On the other hand, even if a fragment of the left side from unique BglII site which is present leftward within the 0.9 kb BamHI - HincII fragment of pAK916 was removed, similar activity to that of the aforementioned BamHI - HincII fragment (pAK916) was observed. It is thus considered that a gene encoding a keto group-introducing enzyme having an enzyme activity for synthesizing canthaxanthin from β -carotene as a substrate is present within the 0.74 kb BglII - HincII fragment of pAK916, and the aforementioned NcoI site is present within this gene. As a result of determining the nucleotide sequence, an open reading frame which corresponds to the gene and has a ribosome binding site just in front of the initiation codon was successfully detected, it was then designated as the crtW gene. The nucleotide sequence of the crtW gene and the encoded amino acid sequence are illustrated in Fig. 1 (SEQ ID NO: 1).

The crtW gene product (CrtW) of *Agrobacterium aurantiacus* sp. nov. MK1 has an enzyme activity for converting a methylene group at the 4-position of a β -ionone ring into a keto group, and one of the specific examples is an enzyme activity for synthesizing canthaxanthin from β -carotene as a substrate by way of echinenone (Example 7 (2); see Fig. 11). Furthermore, the crtW gene product also has an enzyme activity for converting a methylene group at the 4-position of a 3-hydroxy- β -ionone ring into a keto group, and one of the specific examples is an enzyme activity for synthesizing astaxanthin from zeaxanthin as a substrate by way of 4-ketozeaxanthin (Example 7 (1); see Fig. 11). In addition, polypeptides having such enzyme activities and DNA strands encoding these polypeptides have not hitherto been known, and the polypeptides and the DNA strands encoding these polypeptides have no overall homology to any polypeptides or DNA strands having been hitherto known. Also, no such informations have hitherto been described that a methylene group of not only a β -ionone ring and a 3-hydroxy- β -ionone ring but also the other compounds is directly converted into a keto group with an enzyme.

(2) Identification of a hydroxyl group-introducing enzyme gene

Unique SalI site is present within the 2.9 kb BamHI fragment of pAK96. When the 2.9 kb BamHI fragment is cut into two fragments at the SalI site, these two fragments (pAK910 and pAK98) have no hydroxyl group-introducing activity. That is to say, the left fragment (pAK910) has only a keto group-introducing enzyme activity (Example 7 (2)), and the right fragment (pAK98) has only a lycopene-cyclizing enzyme activity (Example 7 (4)). On the other hand, when a 1.4 kb NcoI - KpnI fragment (pAK96NK) containing the aforementioned SalI site is introduced into a β -carotene-producing

Escherichia coli, zeaxanthin is synthesized by way of β -cryptoxanthin (Example 7 (3)). It is thus considered that a gene encoding a hydroxyl group-introducing enzyme which has an enzyme activity for synthesizing zeaxanthin from β -carotene as a substrate is present within the 1.4 kb *Nco*I - *Kpn*I fragment of pAK96NK, and the aforementioned *Sal*I site is present within this gene. As a result of determining the nucleotide sequence, an open reading frame which corresponds to the gene and has a ribosome binding site just in front of the initiation codon was successfully detected, it was then referred to as the *crtZ* gene. The nucleotide sequence of the *crtZ* gene and the encoded amino acid sequence are illustrated in Fig. 2 (SEQ ID NO: 2).

The *crtZ* gene product (CrtZ) of *Agrobacterium aurantiacus* sp. nov. MK1 has an enzyme activity for adding a hydroxyl group to the 3-carbon of a β -ionone ring, and one of the specific examples is an enzyme activity for synthesizing zeaxanthin from β -carotene as a substrate by way of β -cryptoxanthin (Example 7 (3); see Fig. 11). Furthermore, the *crtZ* gene product also has an enzyme activity for adding a hydroxyl group to the 3-carbon of a 4-keto- β -ionone ring, and one of the specific examples is an enzyme activity for synthesizing astaxanthin from canthaxanthin as a substrate by way of phenicoxanthin (Example 6; see Fig. 11). In addition, polypeptides having the latter enzyme activity and DNA strands encoding these polypeptides have not hitherto been known. Also, the CrtZ of *Agrobacterium* showed significant homology to the CrtZ of *Erwinia uredovora* (identity of 57%) at the level of amino acid sequence.

(3) Identification of a lycopene cyclase gene

Astaxanthin can be synthesized from β -carotene with the 2.9 kb *Bam*HI fragment from which a fragment of the right side from a *Kpn*I site had been removed (pAK96K) or with the 2.9 kb *Bam*HI fragment from which a fragment right from the *Pst*I site which is placed further right of the *Kpn*I site had been removed (pAK94) (Example 6), but astaxanthin cannot be synthesized from lycopene. On the other hand, when a 1.6 kb *Sal*I fragment (pAK98), which contains a right fragment from unique *Sal*I site present further left than the aforementioned *Kpn*I site within the 2.9 kb *Bam*HI fragment, was introduced into lycopene-producing *Escherichia coli*, β -carotene was synthesized (Example 7 (4)). It is thus considered that a gene encoding lycopene cyclase that has an enzyme activity for synthesizing β -carotene from lycopene as a substrate is present within the 1.6 kb *Sal*I fragment of pAK98, and this gene is present over a range of the *Kpn*I site and the *Pst*I site. As a result of determining the nucleotide sequence, an open reading frame which corresponds to the gene and has a ribosome binding site just in front of the initiation codon was successfully detected, it was then referred to as the *crtY* gene. The nucleotide sequence of the *crtY* gene and the amino acid sequence to be encoded are illustrated in Figs. 3 - 4 (SEQ ID NO: 3).

The *crtY* gene product (CrtY) of *Agrobacterium aurantiacus* sp. nov. MK1 has significant homology to the CrtY of *Erwinia uredovora* (identity of 44.3%) at the level of amino acid sequence, and the functions of both enzymes are the same.

Example 9: Southern blotting analysis with the chromosomal DNA of the other marine bacteria

Examination was conducted whether a region exhibiting homology with the isolated *crtW* and *crtZ* is obtained from a chromosomal DNAs of the other marine microorganisms. The chromosomal DNAs of *Alcaligenes* sp. PC-1 and *Alteromonas* sp. SD-402 prepared in Example 1 were digested with restriction enzymes *Bam*HI and *Pst*I, and separated by agarose gel electrophoresis. All of the DNA fragments thus separated were denaturated with an alkali solution of 0.5 N NaOH and 1.5 M NaCl, and transferred on a nylon membrane filter over an overnight period. The nylon membrane filter on which DNAs had been adsorbed was dipped in a hybridization solution (6 \times Denhardt, 5 \times SSC, 100 μ g/ml ssDNA), and pre-hybridization was conducted at 60°C for 2 hours. Next, the 1.5 kb DNA fragment cut out from pAK96K with *Bal*I, which contains *crtW* and *crtY*, was labelled with a Mega prime™ DNA labelling systems (Amersham) and [α -³²P]dCTP (~110TBq/mmol) and added to the aforementioned prehybridization solution to conduct hybridization at 60°C for 16 hours.

After hybridization, the filter was washed with 2 \times SSC containing 0.1% SDS at 60°C for 1 hour, and subjected to the detection of signals showing homology by autoradiography. As a result, strong signals were obtained at about 13 kb in the product digested with *Bam*HI and at 2.35 kb in the product digested with *Pst*I in the case of *Alcaligenes* sp. PC-1, and strong signals were obtained at about 5.6 kb in the product digested with *Bam*HI and at 20 kb or more in the product digested with *Pst*I in the case of *Alteromonas* sp. SD-4.

Example 10: Acquisition of a xanthophyll synthesis gene cluster from the other marine bacterium

As it was found from the results of Example 9 that the *Pst*I digest of the chromosomal DNA of *Alcaligenes* sp. PC-1 has a region of about 2.35 kb hybridizing with a DNA fragment containing the *crtW* and *crtZ* genes of *Agrobacterium aurantiacus* sp. nov. MK1, the chromosomal DNA of *Alcaligenes* was digested with *Pst*I, and then DNA fragments of 2 - 3.5 kb in size was recovered by agarose gel electrophoresis. The DNA fragments thus collected were inserted into the *Pst*I site of a vector pBluescript II SK+, and introduced into *Escherichia coli* DH5 α to prepare a partial library of *Alcali-*

genes. When the partial library was subjected to colony hybridization with a 1.5 kb DNA fragment containing the *crtW* and *crtZ* genes of *Agrobacterium* as a probe, a positive colony was isolated from about 5,000 colonies. In this case, colony hybridization was conducted under the same condition as in the Southern blotting analysis shown in Example 9. When plasmid DNA was isolated from the colony thus obtained, and digested with *Pst*I to examine the size of the integrated DNA fragments, it was found that the plasmid contained three different fragments. Thus, a 2.35 kb fragment to be hybridized was selected from the three different DNA fragments by the Southern blotting analysis described in Example 9, the 2.35 kb *Pst*I fragment was recovered by agarose gel electrophoresis and inserted again into the *Pst*I site of pBluescript II SK+ to prepare the plasmids pPC11 and pPC12. In pPC11 and pPC12, the aforementioned 2.35 kb *Pst*I fragment was inserted into the *Pst*I site of pBluescript II SK+ in an opposite direction to each other. The restriction enzyme map of pPC11 is illustrated in Fig. 19.

Example 11: Determination of nucleotide sequence of xanthophyll synthesis gene cluster in *Alcaligenes*

When each of pPC11 and pPC12 was introduced into β -carotene-producing *Escherichia coli*, orange colonies were obtained due to the synthesis of astaxanthin (Example 12) in the former, but no other pigments were newly synthesized in the latter. It was thus considered that the direction of the astaxanthin synthesis gene cluster in the plasmid pPC11 was the same as that of the vector lac promoter. It was also found that pPC11 contained no lycopene cyclizing enzyme genes, since no other pigments were newly produced even if pPC11 was introduced into the lycopene-producing *Escherichia coli*.

It was found that even if a plasmid having a 0.72 kb *Bst*EII - *Eco*RV fragment positioned at the right side of the *Pst*I fragment had been removed (referred to as pPC17, Fig. 19) was introduced into the β -carotene-producing *Escherichia coli*, the transformant of *Escherichia coli* synthesized astaxanthin and the like (Example 12), same as in the case of *E. coli* into which pPC11 was introduced, so that the nucleotide sequence of the 1.63 kb *Pst*I - *Bst*EII fragment in pPC17 was determined.

Deletion mutants were prepared with pPC17 and pPC12 according to the following procedure. A 10 μ g portion of each of pPC17 and pPC12 was digested with *Kpn*I and *Hind*III or *Kpn*I and *Eco*RI, extracted with phenol/chloroform, and DNA was recovered by precipitation with ethanol. Each of DNAs was dissolved in 100 μ l of *Exo*III buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, pH 8.0), 180 units of *Exo*III nuclease was added, and the mixture was maintained at 37°C. A 10 μ l portion was sampled at every 1 minute, and two samples were transferred into a tube in which 20 μ l of an MB buffer (40 mM sodium acetate, 100 mM NaCl, 2 mM ZnCl₂, 10% glycerol, pH 4.5) is contained and which is placed on ice. After completion of the sampling, five tubes thus obtained were maintained at 65°C for 10 minutes to inactivate the enzyme, five units of mung bean nuclease were added, and the mixture was maintained at 37°C for 30 minutes. After the reaction, ten DNA fragments different from each other in the degrees of deletion were recovered for each plasmid by agarose gel electrophoresis. The DNA fragments thus recovered were blunt ended with the Klenow fragment, subjected to the ligation reaction at 16°C overnight, and *Escherichia coli* JM109 was transformed. A single stranded DNA was prepared from each of various clones thus obtained with a helper phage M13K07, and subjected to the sequence reaction with a fluorescent primer cycle-sequence kit available from Applied Biosystem (K.K.), and the DNA sequence was determined with an automatic sequencer.

The DNA sequence comprising 1631 base pairs (bp) thus obtained is illustrated in Figs. 16 - 18 (SEQ ID NO: 7). As a result of examining an open reading frame having a ribosome binding site in front of the initiating codon, two open reading frames which can encode the corresponding proteins (A - B (nucleotide positions 99 - 824 of SEQ ID NO: 7), C - D (nucleotide positions 824 - 1309) in Figs. 16 - 18 were found at the positions where the two xanthophyll synthesis genes *crtW* and *crtZ* were expected to be present.

Example 12: Identification of pigments produced by *Escherichia coli* having an *Alcaligenes* xanthophyll synthesis gene cluster

(1) Identification of astaxanthin and 4-ketozeaxanthin

A deletion plasmid (having only *crtW*) having a deletion from the right *Bst*EII to the nucleotide position 1162 (Fig. 17) (nucleotide position 1162 of SEQ ID NO: 7) among the deletion plasmids from pPC17 prepared in Example 11 was referred to as pPC17-3 (Fig. 19).

The zeaxanthin-producing *Escherichia coli* JM101 (Example 7 (1)) having pPC17-3 introduced therein (pACCAR25 Δ crtX, pPC17-3); exhibiting orange) was cultured in 2 liters of 2YT culture medium containing 150 μ g/ml of Ap and 30 μ g/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, extracted twice with 200 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (15/1). The original orange pigment was separated into three spots at the R_f values of 0.54 (ca. 25%),

0.72 (ca. 30%) and 0.91 (ca. 25%). The pigments at the R_f values of 0.54 and 0.72 were scratched off from the TLC plate, dissolved in a small amount of chloroform/methanol (9/1) or methanol, and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or methanol to give purified materials in a yield of about 1 mg, respectively.

The materials were identified as 4-ketozeaxanthin (R_f 0.54) and astaxanthin (R_f 0.72), since all of the data of their UV-visible, FD-MS spectra and mobility in TLC (developed with chloroform/methanol (15/1)) accorded with those of the standard samples of 4-ketozeaxanthin and astaxanthin. In addition, the pigment at the R_f value of 0.91 was canthaxanthin (Example 12 (2)).

It was also confirmed by the similar analytical procedures that the β -carotene-producing *Escherichia coli* JM101 having pPC11 or pPC17 introduced thereinto (*Escherichia coli* (pACCAR16 Δ crtX, pPC11 or pPC17) (exhibiting orange) produces astaxanthin, 4-ketozeaxanthin and canthaxanthin. Furthermore, it was also confirmed with the authentic sample of phoenicoxanthin obtained in Example 6 that these *E. coli* transformants produce a trace amount of phoenicoxanthin.

(2) Identification of canthaxanthin

The β -carotene-producing *Escherichia coli* JM101 having pPC17-3 introduced thereinto (*Escherichia coli* (pACCAR16 Δ crtX, pPC17-3); exhibiting orange) was cultured in 2 liters of 2YT culture medium containing 150 μ g/ml of Ap and 30 μ g/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, extracted twice with 200 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (50/1). The darkest pigment corresponding to 40% of the total amount of orange pigments was scratched off from the TLC plate, dissolved in a small amount of chloroform/methanol (9/1) or chloroform/methanol (1/1), and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or chloroform/methanol (1/1) to give a purified material in a yield of 2 mg.

The material was identified as canthaxanthin, since all of the data of its UV-visible, FD-MS (m/e 564) spectra and mobility in TLC (developed with chloroform/methanol (50/1)) accorded with those of the standard sample of canthaxanthin (manufactured by BASF). In addition, the pigment of which amount corresponds to 50% of the total amount of the orange pigments observed in the initial extract was considered to be echinenone from its UV-visible spectrum, mobility in silica gel TLC (developed with chloroform/methanol (50/1)), and mobility in HPLC with NOVA PACK HR 6 μ C18 (3.9 × 300 mm; manufactured by Waters) (developed with acetonitrile/methanol/2-propanol (90/6/4)) (Example 7 (2)). In addition, the balance of the extracted pigments, 10%, was unreacted β -carotene.

(3) Identification of zeaxanthin

A plasmid having a 1.15 kb *S*all fragment within pPC11 inserted in the same direction as the plasmid pPC11 into the *S*all site of pBluescript II SK+ was prepared (referred to as pPC13, see Fig. 19).

The β -carotene-producing *Escherichia coli* JM101 having pPC13 introduced thereinto (*Escherichia coli* (pACCAR16 Δ crtX, pPC13); exhibiting yellow) was cultured in 2 liters of 2YT culture medium containing 150 μ g/ml of Ap and 30 μ g/ml of Cm at 37°C for 18 hours. Bacterial cells collected from the culture solution was extracted with 300 ml of acetone, concentrated, extracted twice with 200 ml of chloroform/methanol (9/1), and concentrated to dryness. Then, thin layer chromatography (TLC) was conducted by dissolving the residue in a small amount of chloroform/methanol (9/1) and developing on a silica gel plate for preparative TLC manufactured by Merck with chloroform/methanol (9/1). The darkest pigment corresponding to 90% of the total amount of orange pigments was scratched off from the TLC plate, dissolved in a small amount of chloroform/methanol (9/1) or methanol, and chromatographed on a Sephadex LH-20 column (15 × 300 mm) with an eluent of chloroform/methanol (9/1) or methanol to give a purified material in a yield of 3 mg.

The material was identified as zeaxanthin, since all of the data of its UV-visible, FD-MS (m/e 568) spectra and mobility in TLC (developed with chloroform/methanol (9/1)) accorded with those of the standard sample of zeaxanthin (Example 7 (3)). In addition, the pigment of which amount corresponds to 10% of the total amount of the orange pigments observed in the initial extract was considered to be β -cryptoxanthin from its UV-visible spectrum, mobility in silica gel TLC (developed with chloroform/methanol (9/1)), and mobility in HPLC with NOVA PACK HR 6 μ C18 (3.9 × 300 mm; manufactured by Waters) (developed with acetonitrile/methanol/2-propanol (90/6/4)) (Example 7 (3)).

Example 13: Identification of the *Alcaligenes xanthophyll* synthesis gene cluster

(1) Identification of a keto group-introducing enzyme gene

It is apparent from the results of Examples 11 and 12 (1) that all of the genes required for the synthesis of astaxanthin from β -carotene among the 2.35 kb PstI fragment contained in pPC11 is contained in the 1.63 kb PstI - BstEII fragment (pPC17, Fig. 19) in the left side. Thus, the 0.72 kb BstEII - PstI fragment in the right side is not needed. Unique SmaI and Sall sites are present within the 1.63 kb PstI - BstEII fragment of pPC17 (Fig. 19). It is confirmed by the pigment analysis with a β -carotene-producing *Escherichia coli* having the deletion plasmids introduced therein that the keto group-introducing enzyme activity was lost when the 0.65 kb and 0.69 kb fragments at the left side from SmaI and Sall sites were removed. It was also confirmed by the pigment analysis with a β -carotene-producing *Escherichia coli* having the plasmid introduced therein that the plasmid having a 0.69 kb PstI - Sall fragment positioned at the left side of the 1.63 kb PstI - BstEII fragment inserted into the PstI - Sall site of pBluescript SK+ has no keto group-introducing enzyme activity. On the other hand, the deletion plasmid pPC17-3 (Fig. 19) in which deletion from the BstEII end at the right end to the nucleotide No. 1162 (nucleotide position 1162 in SEQ ID NO: 7) occurred has a keto group-introducing enzyme activity (Example 12 (1), (2)), so that it is considered a gene encoding a keto group-introducing enzyme having an enzyme activity for synthesizing canthaxanthin or astaxanthin with a substrate of β -carotene or zeaxanthin is present in the 1162 bp fragment in pPC17-3, and the aforementioned SmaI and Sall sites are present within this gene. As a result of determining the nucleotide sequence, an open reading frame which corresponds to the gene and has a ribosome binding site just in front of the initiation codon was successfully detected, so that it was referred to as the crtW gene. The nucleotide sequence of the crtW gene and the encoded amino acid sequence are illustrated in Figs. 13 - 14 (SEQ ID NO: 5).

The crtW gene product (CrtW) of *Alcaligenes* sp. PC-1 has an enzyme activity for converting a methylene group at the 4-position of a β -ionone ring into a keto group, and one of the specific examples is an enzyme activity for synthesizing canthaxanthin from β -carotene as a substrate by way of echinenone (Example 12 (2); see Fig. 11). Furthermore, the crtW gene product also has an enzyme activity for converting a methylene group at the 4-position of a 3-hydroxy- β -ionone ring into a keto group, and one of the specific examples is an enzyme activity for synthesizing astaxanthin from zeaxanthin as a substrate by way of 4-ketozeaxanthin (Example 12 (1); see Fig. 11). In addition, polypeptides having such enzyme activities and DNA strands encoding these polypeptides have not hitherto been known, and the polypeptides and the DNA strands encoding these polypeptides have no total homology to any polypeptides or DNA strands having been hitherto known. Also, the crtW gene products (CrtW) of *Agrobacterium aurantiacus* sp. nov. MK1 and *Alcaligenes* sp. PC-1 share high homology (identity of 83%) at the level of amino acid sequence, and the functions of both enzymes are the same. The amino acid sequence in the region of 17% having no identity among these amino acid sequences is considered not so significant to the functions of the enzyme. It is thus considered particularly in this region that a little amount of substitution by the other amino acids, deletion, or addition of the other amino acids will not affect the enzyme activity.

It can be said the keto group-introducing enzyme gene crtW of marine bacteria encodes the β -ionone or 3-hydroxy- β -ionone ring ketolase which converts directly the methylene group at the 4-position into a keto group irrespective to whether a hydroxyl group is added to the 3-position or not. In addition, no such informations have hitherto been described that a methylene group of not only a β -ionone ring and a 3-hydroxy- β -ionone ring but also the other compounds is directly converted into a keto group with one enzyme.

(2) Identification of a hydroxyl group-introducing enzyme gene

All of the genes required for the synthesis of astaxanthin from β -carotene is contained in the 1.63 kb PstI - BstEII fragment (Fig. 19) of pPC17. One Sall site is present within the 1.63 kb PstI - BstEII fragment of pPC17. It is apparent from the results of Example 12 (3) that a hydroxyl group-introducing enzyme activity is present in a fragment at the right side from the Sall site. It is thus understood that the hydroxyl group-introducing enzyme activity is present in the 0.94 kb Sall - BstEII fragment which is the right fragment in the 1.63 kb PstI - BstEII fragment. As a result of determining the nucleotide sequence, an open reading frame which corresponds to the gene and has a ribosome binding site just in front of the initiation codon was successfully detected, it was referred to as the crtZ gene. The nucleotide sequence of the crtZ gene and the encoded amino acid sequence are illustrated in Fig. 15 (SEQ ID NO: 6).

The crtZ gene product (CrtZ) of *Alcaligenes* sp. PC-1 has an enzyme activity for adding a hydroxyl group to the 3-carbon of a β -ionone ring, and one of the specific examples is an enzyme activity for synthesizing zeaxanthin from β -carotene as a substrate by way of β -cryptoxanthin (Example 12 (3); see Fig. 11). Furthermore, the crtZ gene product also has an enzyme activity for adding a hydroxyl group to the 3-carbon of a 4-keto- β -ionone ring, and one of the specific examples is an enzyme activity for synthesizing astaxanthin from canthaxanthin as a substrate by way of phenixanthin (Example 12 (1); see Fig. 11). In addition, polypeptides having the latter enzyme activity and DNA strands encoding these polypeptides have not hitherto been known. Also, the CrtZ of *Alcaligenes* sp. PC-1 showed significant

homology to the CrtZ of Erwinia uredovora (identity of 58%) at the level of amino acid sequence. In addition, the crtZ gene products (CrtZ) of Agrobacterium aurantiacus sp. nov. MK1 and Alcaligenes sp. PC-1 have high homology (identity of 90%) at the level of amino acid sequence, and the functions of both enzymes are the same. The amino acid sequence in the region of 10% having no identity among these amino acid sequences is considered not so significant to the functions of the enzyme. It is thus considered particularly in this region that a little amount of substitution by the other amino acids, deletion, or addition of the other amino acids will not affect the enzyme activity.

(3) Consideration on minor biosynthetic pathways of xanthophylls

It has been elucidated by our studies with carotenoid synthesis genes of the epiphytic bacterium Erwinia or the photosynthetic bacterium Rhodobacter that carotenoid biosynthesis enzymes generally act by recognizing the half of a carotenoid molecule as a substrate. By way of example, the lycopene cyclase gene of Erwinia, crtY, recognizes the halves of the lycopene molecule to cyclize it. When the phytoene desaturase gene crtI of Rhodobacter was used for the synthesis of neurosporene in place of lycopene in Escherichia coli and crtY of Erwinia was allowed to work on it, the crtY gene product recognizes the half molecular structure common to lycopene to produce a half cyclized β -zeacarotene (Linden, H., Misawa, N., Chamovits, D., Pecher, I., Hirschberg, J., Sandmann, G., "Functional Complementation in Escherichia coli of Different Phytoene Desaturase Genes and Analysis of Accumulated Carotenes", Z. Naturforsch., 46c, p. 1045-1051, 1991). Also, in the present invention, when CrtW is allowed to work on β -carotene or zeaxanthin, echinenone or 4-ketozeaxanthin in which one keto group has been introduced is first synthesized, and when CrtZ is allowed to work on β -carotene or canthaxanthin, β -cryptoxanthin or phoenicoxanthin in which one hydroxyl group has been introduced is first synthesized. It can be considered because these enzymes recognize the half molecule of the substrate. Thus, while Escherichia coli having the crtE, crtB, crtI and crtY genes of Erwinia and the crtZ gene of a marine bacterium produces zeaxanthin as described above, β -cryptoxanthin which is β -carotene having one hydroxyl group introduced therein can be detected as an intermediate metabolite. It can be thus considered that if CrtW is present, 3'-hydroxyechinenone or 3-hydroxyechinenone can be synthesized from β -cryptoxanthin as a substrate, and that phoenicoxanthin can be further synthesized by the action of CrtW on these intermediates. The present inventors have not identified these ketocarotenoids in the culture solutions, and the reason is considered to be that only a trace amount of these compounds is present under the conditions carried out in the present experiments. In fact, it was described that 3'-hydroxyechinenone or 3-hydroxyechinenone was detected as a minor intermediate metabolite of astaxanthin in a marine bacterium Agrobacterium aurantiacus sp. nov. MK1 as a gene source (Akihiro Yokoyama ed., "For the biosynthesis of astaxanthin in marine bacteria", Nippon Suisan Gakkai, Spring Symposium, 1994, Abstract, p. 252, 1994). It can be considered from the above descriptions that minor metabolic pathways shown in Fig. 20 are also present in addition to the main metabolic pathways of astaxanthin shown in Fig. 11.

Industrial Applicability

According to the present invention, the gene clusters required for the biosynthesis of keto group-containing xanthophylls such as astaxanthin, phoenicoxanthin, 4-ketozeaxanthin, canthaxanthin and echinenone have successfully been obtained from marine bacteria, and their structures, nucleotide sequences, and functions have been elucidated. The DNA strands according to the present invention are useful as genes capable of affording the ability of biosynthesis of keto group-containing xanthophylls such as astaxanthin to microorganisms such as Escherichia coli and the like.

SEQUENCE LISTING

SEQ ID NO: 1

SEQUENCE LENGTH: 639

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Agrobacterium aurantiacus

STRAIN: sp. nov. MK1

SEQUENCE

GTG CAT GCG CTG TGG TTT CTG GAC GCA GCG GCG CAT CCC ATC CTG GCG 48

Met His Ala Leu Trp Phe Leu Asp Ala Ala Ala His Pro Ile Leu Ala

1 5 10 15

ATC GCA AAT TTC CTG GGG CTG ACC TGG CTG TCG GTC GGA TTG TTC ATC 96

Ile Ala Asn Phe Leu Gly Leu Thr Trp Leu Ser Val Gly Leu Phe Ile

20 25 30

ATC GCG CAT GAC GCG ATG CAC GGG TCG GTG GTG CCG GGG CGT CCG CGC 144

Ile Ala His Asp Ala Met His Gly Ser Val Val Pro Gly Arg Pro Arg

35 40 45

GCC AAT GCG GCG ATG GGC CAG CTT GTC CTG TGG CTG TAT GCC GGA TTT 192

Ala Asn Ala Ala Met Gly Gln Leu Val Leu Trp Leu Tyr Ala Gly Phe

50 55 60

TCG TGG CGC AAG ATG ATC GTC AAG CAC ATG GCC CAT CAC CGC CAT GCC . 240
 Ser Trp Arg Lys Met Ile Val Lys His Met Ala His His Arg His Ala
 65 70 75 80
 GGA ACC GAC GAC GAC CCC GAT TTC GAC CAT CGC GGC CCG GTC CGC TGG 288
 Gly Thr Asp Asp Asp Pro Asp Phe Asp His Gly Gly Pro Val Arg Trp
 85 90 95
 TAC GCC CGC TTC ATC GGC ACC TAT TTC GGC TGG CGC GAG GGG CTG CTG 336
 Tyr Ala Arg Phe Ile Gly Thr Tyr Phe Gly Trp Arg Glu Gly Leu Leu
 100 105 110
 CTG CCC GTC ATC GTG ACC GTC TAT GCG CTG ATC CTT GGG GAT CGC TCG 384
 Leu Pro Val Ile Val Thr Val Tyr Ala Leu Ile Leu Gly Asp Arg Trp
 115 120 125
 ATG TAC GTG GTC TTC TGG CCG CTG CCG TCG ATC CTG GCG TCG ATC CAG 432
 Met Tyr Val Val Phe Trp Pro Leu Pro Ser Ile Leu Ala Ser Ile Gln
 130 135 140
 CTG TTC GTG TTC GGC ACC TGG CTG CCG CAC CGC CCC GGC CAC GAC GCG 480
 Leu Phe Val Phe Gly Thr Trp Leu Pro His Arg Pro Gly His Asp Ala
 145 150 155 160
 TTC CCG GAC CGC CAC AAT GCG CCG TCG TCG CCG ATC AGC GAC CCC GTG 528
 Phe Pro Asp Arg His Asn Ala Arg Ser Ser Arg Ile Ser Asp Pro Val
 165 170 175
 TCG CTG CTG ACC TGC TTT CAC TTT GGC GGT TAT CAT CAC GAA CAC CAC 576
 Ser Leu Leu Thr Cys Phe His Phe Gly Gly Tyr His His Glu His His
 180 185 190

CTG CAC CCG ACG GTG CCG TGG TGG CGC CTG CCC AGC ACC CGC ACC AAG 624

5 Leu His Pro Thr Val Pro Trp Trp Arg Leu Pro Ser Thr Arg Thr Lys

195

200

205

10 GGG GAC ACC GCA TGA 639

Gly Asp Thr Ala ***

15 210

20

25

30

35

40

45

50

55

SEQ ID NO: 2

SEQUENCE LENGTH: 489

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Agrobacterium aurantiacus

STRAIN: sp. nov. MK1

SEQUENCE

ATG ACC AAT TTC CTG ATC GTC GTC GCC ACC GTG CTG GTG ATG GAG TTG 48

Met Thr Asn Phe Leu Ile Val Val Ala Thr Val Leu Val Met Glu Leu

1 5 10 15

ACG GCC TAT TCC GTC CAC CGC TGG ATC ATG CAC GGC CCC CTG GGC TGG 96

Thr Ala Tyr Ser Val His Arg Trp Ile Met His Gly Pro Leu Gly Trp

20 25 30

GGC TGG CAC AAG TCC CAC CAC GAG GAA CAC GAC CAC GCG CTG GAA AAG 144

Gly Trp His Lys Ser His His Glu Glu His Asp His Ala Leu Glu Lys

35 40 45

AAC GAC CTG TAC GGC CTG GTC TTT GCG GTG ATC GCC ACG GTG CTG TTC 192

Asn Asp Leu Tyr Gly Leu Val Phe Ala Val Ile Ala Thr Val Leu Phe

50 55 60

5 ACG GTG GGC TGG ATC TGG GCG CCG GTC CTG TGG TGG ATC GCC TTG GGC 240
 Thr Val Gly Trp Ile Trp Ala Pro Val Leu Trp Trp Ile Ala Leu Gly
 65 70 75 80
 10 ATG ACT GTC TAT GGG CTG ATC TAT TTC GTC CTG CAT GAC GGG CTG GTG 288
 Met Thr Val Tyr Gly Leu Ile Tyr Phe Val Leu His Asp Gly Leu Val
 85 90 95
 15 CAT CAG CGC TGG CCG TTC CGT TAT ATC CCG CGC AAG GGC TAT GCC AGA 336
 His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys Gly Tyr Ala Arg
 100 105 110
 20 CGC CTG TAT CAG GCC CAC CGC CTG CAC CAT GCG GTC GAG GGG CGC GAC 384
 Arg Leu Tyr Gln Ala His Arg Leu His His Ala Val Glu Gly Arg Asp
 115 120 125
 25 CAT TGC GTC AGC TTC GGC TTC ATC TAT GCG CCC CCG GTC GAC AAG CTG 432
 His Cys Val Ser Phe Gly Phe Ile Tyr Ala Pro Pro Val Asp Lys Leu
 130 135 140
 30 AAG CAG GAC CTG AAG ATG TCG GGC GTG CTG CGG GCC GAG GCG CAG GAG 480
 Lys Gln Asp Leu Lys Met Ser Gly Val Leu Arg Ala Glu Ala Gln Glu
 145 150 155 160
 35 CGC ACG TGA 489
 Arg Thr ---
 50 ***
 55

SEQ ID NO: 3

SEQUENCE LENGTH: 1161

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Agrobacterium aurantiacus

STRAIN: sp. nov. MK1

SEQUENCE

GTG ACC CAT GAC GTG CTG CTG GCA GGG GCG GGC CTT GCC AAC GGG CTG 48

Met Thr His Asp Val Leu Leu Ala Gly Ala Gly Leu Ala Asn Gly Leu

1 5 10 15

ATC GCC CTG GCG CTG CGC GCG GCG CGG CCC GAC CTG CGC GTG CTG CTG 96

Ile Ala Leu Ala Leu Arg Ala Ala Arg Pro Asp Leu Arg Val Leu Leu

20 25 30

CTG GAC CAT GCC GCA GGA CCG TCA GAC GGC CAC ACC TGG TCC TGC CAC 144

Leu Asp His Ala Ala Gly Pro Ser Asp Gly His Thr Trp Ser Cys His

35 40 45

GAC CCC GAC CTG TCG CCG GAC TGG CTG GCG CGG CTG AAG CCC CTG CGC 192

Asp Pro Asp Leu Ser Pro Asp Trp Leu Ala Arg Leu Lys Pro Leu Arg

50 55 60

	CGC GCC AAC TGG CCC GAC CAG GAG GTG CGC TTT CCC CGC CAT GCC CGG	240
5	Arg Ala Asn Trp Pro Asp Gln Glu Val Arg Phe Pro Arg His Ala Arg	
	65 70 75 80	
10	CGG CTG GCC ACC GGT TAC GGG TCG CTG GAC GGG GCG GCG CTG GCG GAT	288
	Arg Leu Ala Thr Gly Tyr Gly Ser Leu Asp Gly Ala Ala Leu Ala Asp	
	85 90 95	
15	GCG GTG GTC CGG TCG GGC GCC GAG ATC CGC TGG GAC AGC GAC ATC GCC	336
	Ala Val Val Arg Ser Gly Ala Glu Ile Arg Trp Asp Ser Asp Ile Ala	
20	100 105 110	
	CTG CTG GAT GCG CAG GGG GCG ACG CTG TCC TGC GGC ACC CGG ATC GAG	384
25	Leu Leu Asp Ala Gln Gly Ala Thr Leu Ser Cys Gly Thr Arg Ile Glu	
	115 120 125	
30	GCG GCC GCG GTC CTG GAC GGG CGG GGC GCG CAG CCG TCG CGG CAT CTG	432
	Ala Gly Ala Val Leu Asp Gly Arg Gly Ala Gln Pro Ser Arg His Leu	
	130 135 140	
35	ACC GTG GGT TTC CAG AAA TTC GTG GGT GTC GAG ATC GAG ACC GAC CGC	480
	Thr Val Gly Phe Gln Lys Phe Val Gly Val Glu Ile Glu Thr Asp Arg	
40	145 150 155 160	
	CCC CAC GGC GTG CCC CGC CCG ATG ATC ATG GAC GCG ACC GTC ACC CAG	528
	Pro His Gly Val Pro Arg Pro Met Ile Met Asp Ala Thr Val Thr Gln	
45	165 170 175	
	CAG GAC GGG TAC CGC TTC ATC TAT CTG CTG CCC TTC TCT CCG ACG CGC	576
50	Gln Asp Gly Tyr Arg Phe Ile Tyr Leu Leu Pro Phe Ser Pro Thr Arg	
	180 185 190	
55		

5 ATC CTG ATC GAG GAC ACG CGC TAT TCC GAT GGC GGC GAT CTG GAC GAC 624
 Ile Leu Ile Glu Asp Thr Arg Tyr Ser Asp Gly Gly Asp Leu Asp Asp
 195 200 205
 10 GAC GCG CTG GCG GCG GCG TCC CAC GAC TAT GCC CGC CAG CAG GGC TGG 672
 Asp Ala Leu Ala Ala Ala Ser His Asp Tyr Ala Arg Gln Gln Gly Trp
 210 215 220
 15 ACC GGG GCC GAG GTC CGG CGC GAA CGC GGC ATC CTT CCC ATC GCG CTG 720
 Thr Gly Ala Glu Val Arg Arg Glu Arg Gly Ile Leu Pro Ile Ala Leu
 225 230 235 240
 20 GCC CAT CAT GCG GCG GCG TTC TGG GCC GAT CAC GCG GCG GGG CCT GTT 768
 Ala His Asp Ala Ala Gly Phe Trp Ala Asp His Ala Ala Gly Pro Val
 245 250 255
 30 CCC GTG GGA CTG CGC GCG GGG TTC TTT CAT CCG GTC ACC GGC TAT TCG 816
 Pro Val Gly Leu Arg Ala Gly Phe Phe His Pro Val Thr Gly Tyr Ser
 260 265 270
 35 CTG CCC TAT GCG GCA CAG GTG GCG GAC GTG GTG GCG GGT CTG TCC GGG 864
 Leu Pro Tyr Ala Ala Gln Val Ala Asp Val Val Ala Gly Leu Ser Gly
 275 280 285
 40 CCG CCC GGC ACC GAC GCG CTG CGC GGC GCC ATC CGC GAT TAC GCG ATC 912
 Pro Pro Gly Thr Asp Ala Leu Arg Gly Ala Ile Arg Asp Tyr Ala Ile
 290 295 300
 50 GAC CGG GCG CGC CGC GAC CGC TTT CTG CGC CTT TTG AAC CGG ATG CTG 960
 Asp Arg Ala Arg Arg Asp Arg Phe Leu Arg Leu Leu Asn Arg Met Leu
 305 310 315 320
 55

TTC CGC GGC TGC GCG CCC GAC CGG CGC TAT ACC CTG CTG CAG CGG TTC 1008

Phe Arg Gly Cys Ala Pro Asp Arg Arg Tyr Thr Leu Leu Gln Arg Phe

325

330

335

TAC CGC ATG CCG CAT GGA CTG ATC GAA CGG TTC TAT GCC GGC CGG CTG 1056

Tyr Arg Met Pro His Gly Leu Ile Glu Arg Phe Tyr Ala Gly Arg Leu

340

345

350

AGC GTG GCG GAT CAG CTG CGC ATC GTG ACC GGC AAG CCT CCC ATT CCC 1104

Ser Val Ala Asp Gln Leu Arg Ile Val Thr Gly Lys Pro Pro Ile Pro

355

360

365

CTT GGC ACG GCC ATC CGC TGC CTG CCC GAA CGT CCC CTG CTG AAG GAA 1152

Leu Gly Thr Ala Ile Arg Cys Leu Pro Glu Arg Pro Leu Leu Lys Glu

370

375

380

AAC GCA TGA

1161

Asn Ala ***

385

SEQ ID NO: 4

SEQUENCE LENGTH: 2886

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Agrobacterium aurantiacus

STRAIN: sp. nov. MK1

SEQUENCE

GGATCCGGCG ACCTTGCGGC GCTGCGCGCG CGCCCTTTGC TGGTGCCTGG GCCGGGTGGC 60

CCTAGGCCGC TGGAACGCCG CGACGCGGCG CGCGGAAACG ACCACGGACC CGGCCACCG

CAATGGTCGC AAGCAACGGG GATGGAACCG GCGGATGCGG GACTGTAGTC TCGCGGATC 120

GTTACCAGCG TTCGTTGCCC CTACCTTTGG CCGCTACGCC CTGACATCAG ACGCGCCTAG

GCCGGTCCGG GGGACAAGAT GAGCGCACAT GCCCTGCCCC AGGCAGATCT GACCGCCACC 180

CGGCCAGGCC CCCTGTTCTA CTCGCGTGTA CCGGACGGGT TCCGTCTAGA CTGGCGGTGG

AGCCTGATCG TCTCGGGCGG CATCATCGCC GCTTGGCTGG CCCTGCATGT GCATGCGCTG 240

TCGGACTAGC AGAGCCCGCC GTAGTAGCGG CGAACCGACC GGGACGTACA CGTACCGGAC

TGGTTTCTGG ACGCAGCGGC GCATCCCATC CTGGCGATCG CAAATTTCTT GGGGCTGACC 300

ACCAAAGACC TCGCTCGCCG CGTAGGGTAG GACCGCTAGC GTTTAAAGGA CCCCAGCTGG

TGGCTGTCGG TCGGATTGTT CATCATCGCG CATGACGGGA TGCACGGGTC GGTGGTGCCG . 360

ACCGACAGCC AGCCTAACAA GTAGTAGCGC GTACTGCGCT ACGTGCCCAG CCACCACGGC

GGGCGTCCGC GCGCCAATGC GCGGATGGGC CAGCTTGTCG TGTGGCTGTA TGCCGGATTT 420

CCCGCAGGCG CGCGGTACG CCGCTACCCG GTCGAACAGG ACACCGACAT ACGGCCTAAA

TGGTGGCGCA AGATGATCGT CAAGCACATG GCCCATCACC GCCATGCCGG AACCGACGAC 480

AGCACCGCGT TCTACTAGCA GTTCGTGTAC CCGGTAGTGG CGGTACGGCC TTGGCTGCTG

GACCCCGATT TCGACCATGG CCGCCCCGGTC CGCTGGTACG CCGGCTTCAT CGGCACCTAT 540

CTGGGGCTAA AGCTGGTACC GCGGGGCCAG GCGACCATGC GGGCGAAGTA GCGGTGGATA

TTGGGCTGGC GCGAGGGGCT GCTGCTGCCC GTCATCGTGA CCGTCTATGC GCTGATCCTT 600

AAGCCGACCG CGTCCCCGA CGACGACGGG CAGTAGCACT GCCAGATACG CGACTAGGAA

GGGGATCGCT GGATGTACCT GGTCTTCTGG CCGCTGCCGT CGATCCTGGC GTCGATCCAG 660

CCCCTAGCGA CCTACATGCA CCAGAAGACC GCGGACGGCA GCTAGGACCG CAGCTAGGTC

CTGTTCTGT TCGGCACCTG GCTCCCGCAC CGCCCCGGCC ACGACGCGTT CCCGGACCGC 720

GACAAGCACA AGCCGTGGAC CGACGGCGTG GCGGGGCCGG TGCTGCGCAA GGGCCTGGCG

CACAATGCGC GGTGCTCGCG GATCAGCGAC CCCGTGTGCG TGCTGACCTG CTTTCACTTT 780

TGTTACGCG CCAGCAGCGC CTAGTCGCTG GGGCACAGCG ACGACTGGAC GAAAGTGAAA

GGCGGTATC ATCACGAACA CCACCTGCAC CCGACGGTGC CGTGGTGGCG CCTGCCCAGC 840

CCGCCAATAG TAGTGCTTGT GGTGGACGTG GGCTGCCACG GCACCACCGC GGACGGGTGC

ACCCGCACCA AGGGGGACAC CGCATGACCA ATTTCTGAT CGTCGTGGCC ACCGTGCTGG 900

TGGGCGTGGT TCCCCCTGTG GCGTACTGGT TAAAGGACTA GCAGCAGCGG TGGCACCACC

TGATGGAGTT GACGGCCTAT TCCGTCCACC GCTGGATCAT GCACGGCCCC CTGGGCTGGG 960

ACTACCTCAA CTGCCGGATA AGGCAGGTGG CGACCTAGTA CGTGCCGGGG GACCCGACCC

GCTGGCACAA GTCCCACCAC GAGGAACACG ACCACGGCT GGAAAAGAAC GACCTGTACG 1020

CGACCGTGTT CAGGGTGGTG CTCCTTGTGC TGGTCCGCGA CCTTTTCTTG CTGGACATGC

GCCTGGTCTT TCGGTGATC GCCACGGTGC TGTTACGGT GGGCTGGATC TGGGCGCCGG 1080

CGGACCAGAA ACGCCACTAG CCGTGCCACG ACAAGTGCCA CCGACCTAG ACCCGCGGCC

TCCTGTGGTG GATCGCCTTG GGCATGACTG TCTATGGGT GATCTATTTG GTCCTGCATG 1140

AGGACACCAC CTAGCGGAAC CCGTACTGAC AGATACCCGA CTAGATAAAG CAGGACGTAC

ACGGGCTGGT GCATCAGCGC TGGCCCTTCC GTTATATCCC GCGCAAGGGC TATGCCAGAC 1200

TGCCCCACCA CGTAGTCGGC ACCGGCAAGG CAATATAGGG CCGGTTCCCG ATACGGTCTG

GCCTGTATCA GGCCCACCGC CTGCACCATG CGGTGAGGG GCGCGACCAT TGCGTCAGCT 1260

CGGACATAGT CCGGGTGGCG GACGTGGTAC GCCAGCTCCC CCGGCTGGTA ACGCAGTCGA

TCGGCTTCAT CTATGCGCCC CCGGTCGACA AGCTGAAGCA GGACCTGAAG ATGTGGGGCG 1320
 5 AGCCGAAGTA GATACGCGGG GGCCAGCTGT TCGACTTCGT CCTGGACTTC TACAGCCCGC

10 TGCTGCGGGC CGAGGCGCAG GAGCGCACGT GACCCATGAC GTGCTGCTGG CAGGGGCGGG 1380
 ACGACGCCCC GCTCCGCGTC CTCGCGTGCA CTGGGTACTG CACGACGACC GTCCCCGGCC

15 CCTTGCCAAC GGGCTGATCG CCCTGGCGCT GCGCGCGCGC CGGCCCCGACC TCGCGGTGCT 1440
 GGAACGGTTG CCCGACTAGC GGGACCGCGA CCGCGCGCGC GCGGGGCTGG ACGCGCACGA

20 GCTGCTGGAC CATGCCGCGAG GACCGTCAGA CGGCCACACC TGGTCCTGCC ACGACCCCGA 1500
 25 CGACGACCTG GTACGCGCTC CTGGCAGTCT GCGGGTGTGG ACCAGGACGG TGCTGGGGCT

30 CCTGTGCGCG GACTGGCTGG CGCGGCTGAA GCCCCTGCGC CGCGCCAACCT GGCCCCACCA 1560
 GGACAGCGGC CTGACCGACC GCGCGGACTT CCGGGACCGG GCGCGGTTGA CCGGGCTGGT

35 GGAGGTGCGC TTTCCCCGCC ATGCCCGGCG GCTGGCCACC GGTACGGGT CGCTGGACGG 1620
 CCTCCACGCG AAAGGGGCGG TACGGGGCCG CGACCGGTGG CCAATGCCCA GCGACCTGCC

40 GCGGGCGCTG GCGGATGCGG TGGTCCGGTC GGGCGCCGAG ATCCGCTGGG ACAGCGACAT 1680
 45 CCGCCGCGAC CGCCTACGCC ACCAGGCCAG CCGCGGGCTC TAGGCGACCC TGTCGCTGTA

50 CGCCCTGCTG GATGCGCAGG GGGCGACGCT GTCTGCGGC ACCCGGATCG AGGCGGGCGC 1740
 GCGGGACGAC CTACGCGTCC CCCGCTGCGA CAGGACGCGG TGGGCCTAGC TCCGCCCGCG

GGTCCTGGAC GGGCGGGGCG CGCAGCCGTC GCGGCATCTG ACCGTGGGTT TCCAGAAAT 1800

5 CCAGGACCTG CCGCGCCGCG GCGTCGGCAG CGCCGTAGAC TGGCACCCAA AGGTCTTTAA

10 CGTGGGTGTC GAGATCGAGA CCGACCGCCC CCACGGCGTG CCGCGCCCGA TGATCATGGA 1860

GCACCCACAG CTCTAGCTCT GGCTGGCGGG GGTGCCGCAC GGGCGGGCT ACTAGTACCT

15 CCGGACCGTC ACCCAGCAGG ACGGGTACCG CTTTATCTAT CTGCTGCCCT TCTCTCCGAC 1920

CGGCTGGCAG TGGGTGCTCC TGCCCATGGC GAAGTAGATA GACGACGGGA AGAGAGGCTG

20 CCGCATCCTG ATCGAGGACA CCGGCTATTC CGATGGCGGC GATCTGGACG ACGACGGCT 1980

25 CCGGTAGGAC TAGCTCCTGT GCGCGATAAG GCTACCGCCG CTAGACCTGC TGCTGCGCGA

30 GCGGGCGGCG TCCCACGACT ATGCCCGCCA GCAGGGCTGG ACCGGGGCGG AGGTCCGGCG 2040

CGCGCGCGCG AGGGTGCTGA TACGGGCGGT CGTCCCGACC TGGCCCCGCG TCCAGGCCCG

35 CGAACGCGGC ATCCTTCCCA TCGCGCTGGC CCATGATGCG GCGGGCTTCT GGGCCGATCA 2100

40 GCTTGCGCCG TAGGAAGGGT AGCCCGACCG GGTACTACGC CGCCGAAGA CCGGGCTAGT

CGCGCGGGG CCTGTTCGG TGGGACTGCG CGCGGGGTTT TTTATCCGG TCACCGGCTA 2160

45 GCGCGCGCGG GGACAAGGGC ACCCTGACGC GCGCCCCAAG AAAGTAGGCC AGTGGCCGAT

50 TTGCTGCCC TATGCGGCAC AGGTGCGCGA CGTGGTGGCG GGTCTGTCCG GCGCGCGCGG 2220

55 AAGCGACGGG ATACGCGGTG TCCACCGCCT GCACCACCGC CCAGACAGGC CCGGCGGGCG

CACCGACGGC CTGCGCGCGC CCATCCGCGA TTACGCGATC GACCGGGCGC GCCGCGACCG 2280
5 GTGGCTGCGC GACCGCGCGC GGTAGGCGCT AATGCGCTAG CTGGCCCGCG CGGCGCTGGC

10 CTTTCTGCGC CTTTGAACC GGATGCTGTT CCGCGGCTGC GCGCCCGACC GCGCTATAC 2340
GAAAGACGGC GAAAACTTGG CCTACGACAA GCGCCCGACC CGCGGGCTGG CCGCGATATG

15 CCTGCTGCAG CGGTTCTACC GCATGCCGCA TGGACTGATC GAACGGTTCT ATGCCGGCCG 2400
GGACGACGTC GCCAAGATGG CGTACGGCGT ACCTGACTAG CTTGCCAAGA TACGGCCGGC

20 CGTGAGCGTG GCGGATCAGC TCGGCATCGT GACCGGCAAG CCTCCCATTC CCCTTGGCAC 2460
25 CCACTCGCAC CGCCTAGTGG ACGCGTAGCA CTGGCCGTTC GGAGGGTAAG GGAACCGTG

30 GGCATCCGC TGCCTGCCCC AACGTCCCCT GCTGAAGGAA AACGCATGAA CGCCCATTCG 2520
CCGGTAGGCG ACGGACGGGC TTGCAGGGGA CGACTTCCTT TTGCGTACTT GCGGGTAAGC

35 CCGCGGGCCA AGACCGCCAT CGTGATCGGC GCAGGCTTTG GCGGGCTGGC CCTGGCCATC 2580
40 GCGCGCCGGT TCTGGCGGTA GCACTAGCCG CGTCCGAAAC CGCCCGACCG GGACCGGTAG

45 CCGCTGCAGT CCGCGGGCAT CGCCACCACC CTGGTCGAGG CCGGGGACAA GCCCGGCGGG 2640
GCGGACGTCA GCGCGCCGTA GCGGTGGTGG GACCAGCTCC GGGCCCTGTT CGGGCCGCCC

50 CCGCCCTATG TCTGGCACGA TCAGGGCCAT CTCTTCGACG CCGGCCCCGAC CGTCATCACC 2700
GCCCGGATAC AGACCGTGCT AGTCCCGGTA GAGAAGCTGC GCCCGGGCTG GCAGTAGTGG

GACCCCGATG CGCTGAAAGA GCTGTGGGCC CTGACCGGGC AGGACATGGC GCGCGACGTG 2760

5 CTGGGGCTAC GCGACTTTCT CGACACCCGG CACTGGCCCG TCCTGTACCG CCGGCTGCAC

10 ACGCTGATGC CGGTCTCGCC CTTCTATCGG CTGATGTGGC CCGGCGGGAA GGTCTTCGAT 2820

TGGGACTACG GCCAGAGCGG GAAGATAGCC GACTACACCG GCCCGCCCTT CCAGAAGCTA

15 TACGTGAACG AGGCCGATCC AGGGTCTGGG TCTTGCCGTG CCAGGTGAAG CTGTTGCCGT 2880

20 ATGCACTTGC TCCGGCTAGG TCCCAGACCC AGAACGGCAC GGTCCACTTC GACAACGGCA

25 GGATCC

2886

CCTAGG

SEQ ID NO: 5

SEQUENCE LENGTH: 729

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Alcaligenes

STRAIN: sp. PC-1

SEQUENCE

ATG TCC GGA CGG AAG CCT GGC ACA ACT GGC GAC ACG ATC GTC AAT CTC 48

Met Ser Gly Arg Lys Pro Gly Thr Thr Gly Asp Thr Ile Val Asn Leu

1 5 10 15

GGT CTG ACC GCC GCG ATC CTG CTG TGC TGG CTG GTC CTG CAC GCC TTT 96

Gly Leu Thr Ala Ala Ile Leu Leu Cys Trp Leu Val Leu His Ala Phe

20 25 30

ACG CTA TGG TTG CTA GAT GCG GCC GCG CAT CCG CTG CTT GCC GTG CTG 144

Thr Leu Trp Leu Leu Asp Ala Ala Ala His Pro Leu Leu Ala Val Leu

35 40 45

TGC CTG GCT GGG CTG ACC TGG CTG TCG GTC GGG CTG TTC ATC ATC GCG 192

Cys Leu Ala Gly Leu Thr Trp Leu Ser Val Gly Leu Phe Ile Ile Ala

50 55 60

CAT GAC GCA ATG CAC GGG TCC GTG GTG CCG GGG CCG CCG GCC AAT 240
 His Asp Ala Met His Gly Ser Val Val Pro Gly Arg Pro Arg Ala Asn
 65 70 75 80
 CCG CCG ATC GGG CAA CTG GCG CTG TGG CTC TAT GCG GGG TTC TCG TCG 288
 Ala Ala Ile Gly Gln Leu Ala Leu Trp Leu Tyr Ala Gly Phe Ser Trp
 85 90 95
 CCC AAG CTG ATC GCC AAG CAC ATG ACG CAT CAC CCG CAC GCC GGC ACC 336
 Pro Lys Leu Ile Ala Lys His Met Thr His His Arg His Ala Gly Thr
 100 105 110
 GAC AAC GAT CCC GAT TTC GGT CAC GGA GGG CCC GTG CCG TGG TAC GGC 384
 Asp Asn Asp Pro Asp Phe Gly His Gly Gly Pro Val Arg Trp Tyr Gly
 115 120 125
 AGC TTC GTC TCC ACC TAT TTC GGC TGG CGA GAG GGA CTG CTG CTA CCG 432
 Ser Phe Val Ser Thr Tyr Phe Gly Trp Arg Glu Gly Leu Leu Leu Pro
 130 135 140
 GTG ATC GTC ACC ACC TAT GCG CTG ATC CTG GGC GAT CCG TGG ATG TAT 480
 Val Ile Val Thr Thr Tyr Ala Leu Ile Leu Gly Asp Arg Trp Met Tyr
 145 150 155 160
 GTC ATC TTC TGG CCG GTC CCG GCC GTT CTG GCG TCG ATC CAG ATT TTC 528
 Val Ile Phe Trp Pro Val Pro Ala Val Leu Ala Ser Ile Gln Ile Phe
 165 170 175
 GTC TTC GGA ACT TGG CTG CCC CAC CCG CCG GGA CAT GAC GAT TTT CCC 576
 Val Phe Gly Thr Trp Leu Pro His Arg Pro Gly His Asp Asp Phe Pro
 180 185 190

GAC CGG CAC AAC GCG AGG TCG ACC GGC ATC GGC GAC CCG TTG TCA CTA 624

Asp Arg His Asn Ala Arg Ser Thr Gly Ile Gly Asp Pro Leu Ser Leu

195

200

205

CTG ACC TGC TTC CAT TTC GGC GGC TAT CAC CAC GAA CAT CAC CTG CAT 672

Leu Thr Cys Phe His Phe Gly Gly Tyr His His Glu His His Leu His

210

215

220

CCG CAT GTG CCG TGG TGG CGC CTG CCT CGT ACA CGC AAG ACC GGA GGC 720

Pro His Val Pro Trp Trp Arg Leu Pro Arg Thr Arg Lys Thr Gly Gly

225

230

235

240

CGC GCA TGA

729

Arg Ala***

SEQ ID NO: 6

SEQUENCE LENGTH: 489

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Alcaligenes

STRAIN: sp. PC-1

SEQUENCE

ATG ACG CAA TTC CTC ATT GTC GTG GCG ACA GTC CTC GTG ATG GAG CTG 48

Met Thr Gln Phe Leu Ile Val Val Ala Thr Val Leu Val Met Glu Leu

ACC GCC TAT TCC GTC CAC CGC TGG ATT ATG CAC GGC CCC CTA GGC TGG 96

Thr Ala Tyr Ser Val His Arg Trp Ile Met His Gly Pro Leu Gly Trp

GGC TGG CAC AAG TCC CAT CAC GAA GAG CAC GAC CAC GCG TTG GAG AAG 144

Gly Trp His Lys Ser His His Glu Glu His Asp His Ala Leu Glu Lys

AAC GAC CTC TAC GGC GTC GTC TTC GCG GTG CTG GCG ACG ATC CTC TTC 192

Asn Asp Leu Tyr Gly Val Val Phe Ala Val Leu Ala Thr Ile Leu Phe

55

SEQ ID NO: 7

SEQUENCE LENGTH: 1631

SEQUENCE TYPE: STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Alcaligenes

STRAIN: sp. PC-1

SEQUENCE

CTGCAGCCCG GGGCCGGTGG CCAATGGTCG CAACCGGCAG GACTGGAACA GGACGGCCGG 60
GACGTCCGGC CCGGGCCACC GGTACCAGC GTTGGCCGTC CTGACCTTGT CCTGCCGCCC

CCGGTCTAGG CTGTCCGCTT ACGCAGCAGG AGTTTCGGAT GTCCGGACGG AAGCCTGGCA 120
GGCCAGATCC GACAGCGGGA TCGTCGTCC TAAAAGCCTA CAGGCCTGCC TTCGGACCGT

CAACTGGCGA CACGATCGTC AATCTCGGTC TGACCGCCGC GATCCTGCTG TGCTGGCTGG 180
GTTGACCGCT GTGCTAGCAG TTAGAGCCAG ACTGGCGGCG CTAGGACGAC ACGACCGACC

TCCTGCACGC CTTTACGCTA TGGTTGCTAG ATGCGGCCGC GCATCCGCTG CTTGCCGTGC 240
AGGACGTCCG GAAATGCGAT ACCAACGATC TACGCCGGCG CGTAGGCGAC GAACGGCACG

5 TGTGCCTGGC TGGGCTGACC TGGCTGTCCG TCGGGCTGTT CATCATCGCG CATGACGCAA 300
ACACGGACCG ACCCGACTGG ACCGACAGCC AGCCCGACAA GTAGTAGCGC GTACTGCGTT

10 TGCACGGGTC CGTGGTGCCG GGGCGGCCCG GCGCCAATGC GCGGATCGGG CAACTGGCGC 360
ACGTGCCCAG GCACCACGGC CCGCGCGGCG CGCGGTACG CCGCTAGCCC GTTGACCGCG

15 TGTGGCTCTA TCGGGGGTTC TCGTGGCCCA AGCTGATCGC CAAGCACATG ACGCATCACC 420
ACACCGAGAT ACGCCCCAAG AGCACCAGGT TCGACTAGCG GTTCGTGTAC TCGTAGTGG

20 GGCACGCCCG CACCGACAAC GATCCCGATT TCGGTCACGG AGGGCCCGTG CGCTGGTACG 480
25 CCGTGCGGCC GTGGCTGTTG CTAGGGCTAA AGCCAGTGCC TCCCGGGCAC GCGACCATGC

30 GCAGCTTCGT CTCCACCTAT TTCGGCTGGC GAGAGGGACT GCTGCTACCG GTGATCGTCA 540
CGTCGAAGCA GAGGTGGATA AAGCCGACCG CTCTCCCTGA CGACGATGGC CACTAGCAGT

35 CCACCTATGC GCTGATCCTG GCGGATCGCT GGATGTATGT CATCTTCTGG CCGGTCCCGG 600
GGTGGATACG CGACTAGGAC CCGCTAGCGA CCTACATACA GTAGAAGACC GGCCAGGGCC

40 CCGTTCTGGC GTCGATCCAG ATTTTCGTCT TCGGAACCTG GCTGCCCCAC CGCCCGGGAC 660
45 GGCAAGACCG CAGCTAGGTC TAAAAGCAGA AGCCTTGAAC CGACGGGGTG GCGGGCCCTG

50 ATGACGATTT TCCCGACCGG CACAACCGGA GGTCCACCGG CATCGGCGAC CCGTTGTCAC 720
TACTGCTAAA AGGGCTGGCC GTGTTGCGCT CCAGCTGGCC GTAGCCGCTG GGCAACAGTG

TACTGACCTG CTTCATTTC GCGGGCTATC ACCACGAACA TCACCTGCAT CCGCATGTGC. 780
ATGACTGCAC GAAGGTAAAG CCGCCGATAG TGGTGCTTGT AGTGGACGTA GCGGTACACC

CGTGGTGGCG CCTGCCCTCGT ACACGCAAGA CCGGAGGCCG CGCATGACGC AATTCCTCAT 840
GCACCACCGC GGACCGAGCA TGTGCGTTCT GGCCTCCGGC GCGTACTGCG TTAAGGAGTA

TGTCGTGGCG ACAGTCCTCG TGATGGAGCT GACCGCCTAT TCCGTCCACC GCTGGATTAT 900
ACACGACCGC TGTGAGGAGC ACTACCTCGA CTGGCGGATA AGGCAGGTGG CGACCTAATA

GCACGGCCCC CTAGGCTGGG GCTGGCACAA GTCCCATCAC GAAGAGCAGC ACCACGCCTT 960
CGTGCCGGGG GATCCGACCC CGACCGTGTG CAGGGTAGTG CTTCTCGTGC TGGTGGCGAA

GGAGAAGAAC GACCTCTACG GCGTCGTCTT CGCGGTGCTG CGGACGATCC TCTTCACCGT 1020
CCTCTTCTTG CTGGAGATGC CGCAGCAGAA GCGCCACGAC CGCTGCTAGG AGAAGTGGCA

GGCGCCCTAT TGGTGGCCGG TGCTGTGGTG GATCGCCCTG GGCATGACGG TCTATGGGTT 1080
CCCGCGGATA ACCACCGGCC ACGACACCAC CTAGCGGGAC CCGTACTGCC AGATACCCAA

GATCTATTTC ATCCTGCACG ACGGGCTTGT GCATCAACGC TGGCCGTTTC GGTATATTCC 1140
CTAGATAAAG TAGGACGTGC TGCCCGAACA CGTAGTTGCG ACCGGCAAAG CCATATAAGG

GCGGCGGGGC TATTTCGCA GGCTCTACCA AGCTCATCGC CTGCACCACG CCGTCGAGGG 1200
CGCCGCCCCG ATAAAGGCGT CCGAGATGGT TCGAGTAGCG GACGTGGTGC GCCAGCTCCC

GGGGGACCAC TGGTCAGCT TCGGCTTCAT CTATGCCCCA CCGTGGACA AGCTGAAGCA 1260

5 CCCCCTGGTG ACGCAGTCGA AGCCGAAGTA GATACGGGGT GGGCACCTGT TCGACTTCGT

10 GGATCTGAAG CGGTCCGGTG TCCTCCGCCC CCAGGACGAG CGTCCGTCGT GATCTCTGAT 1320

CCTAGACTTC GCCAGCCCAC AGGACCGGGG GGTCTGCTC GCAGGCAGCA CTAGAGACTA

15 CCGGGCGTGG CCGCATGAAA TCCGACGTGC TCCTGGCAGG GGCCGGCCTT GCCAACGGAC 1380

GGGCGGCACC GGCCTACTTT AGGCTGCACG ACGACCGTCC CCGGCCGGAA CCGTTGCCTG

20 TGATCGCGCT GCGGATCCGC AAGCGCGGGC CCGACCTTCG CGTGCTGCTG CTGGACCGTG 1440

25 ACTAGCGCGA CCGCTAGGCG TTCCGCGCCG GGCTGGAAGC GCACGACGAC GACCTGGCAC

30 CCGCGGGCGC CTCGGACGGG CATACTTGGT CCTGCCACGA CACCGATTTC GCGCCGCACT 1500

GCGGCCGCG GAGCCTGCCC GTATGAACCA GGACGGTGCT GTGGCTAAAC CCGGGCGTGA

35 GGCTGGACCG CCTGAAGCCG ATCAGGCGTG GCGACTGGCC CGATCAGGAG GTGCGGTTCC 1560

40 CCGACCTGGC GGA CTTCGGC TAGTCCGCAC CGCTGACCGG GCTAGTCCTC CACGCCAAGG

45 CAGACCATTC GCGAAGGCTC CCGGCCGGAT ATGGCTCGAT CGACGGGCGG GGGCTGATGC 1620

GTCTGGTAAG CGCTTCCGAG GCGCGGCCTA TACCGAGCTA GCTGCCCCGC CCCGACTACG

50 GTGCGGTGAC C

1631

CACGCCACTG G

Claims

1. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting a methylene group at the 4-position of a β -ionone ring into a keto group.
2. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.
3. A DNA strand hybridizing the DNA strand according to claim 2 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 2.
4. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.
5. A DNA strand hybridizing the DNA strand according to claim 4 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 4.
6. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting β -carotene into canthaxanthin by way of echinenone and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.
7. A DNA strand hybridizing the DNA strand according to claim 6 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 6.
8. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting β -carotene into canthaxanthin by way of echinenone and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.
9. A DNA strand hybridizing the DNA strand according to claim 8 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 8.
10. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the 3-hydroxy- β -ionone ring into a keto group.
11. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the 3-hydroxy- β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.
12. A DNA strand hybridizing the DNA strand according to claim 11 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 11.
13. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting the methylene group at the 4-position of the 3-hydroxy- β -ionone ring into a keto group and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.
14. A DNA strand hybridizing the DNA strand according to claim 13 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 13.
15. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting zeaxanthin into astaxanthin by way of 4-ketozeaxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 212 which is shown in the SEQ ID NO: 1.
16. A DNA strand hybridizing the DNA strand according to claim 15 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 15.

17. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting zeaxanthin into astaxanthin by way of 4-ketozeaxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 242 which is shown in the SEQ ID NO: 5.
18. A DNA strand hybridizing the DNA strand according to claim 17 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 17.
19. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for adding a hydroxyl group to the 3-carbon of the 4-keto- β -ionone ring.
20. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for adding a hydroxyl group to position 3-carbon of the 4-keto- β -ionone ring and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 2.
21. A DNA strand hybridizing the DNA strand according to claim 20 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 20.
22. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for adding a hydroxyl group to position 3-carbon of the 4-keto- β -ionone ring and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 6.
23. A DNA strand hybridizing the DNA strand according to claim 22 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 22.
24. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting canthaxanthin into astaxanthin by way of phoenicoxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 2.
25. A DNA strand hybridizing the DNA strand according to claim 24 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 24.
26. A DNA strand having a nucleotide sequence which encodes a polypeptide having an enzyme activity for converting canthaxanthin into astaxanthin by way of phoenicoxanthin and having an amino acid sequence substantially of amino acid Nos. 1 - 162 which is shown in the SEQ ID NO: 6.
27. A DNA strand hybridizing the DNA strand according to claim 26 and having a nucleotide sequence which encodes a polypeptide having an enzyme activity according to claim 26.
28. A process for producing a xanthophyll comprising introducing the DNA strand according to any one of claims 1 - 9 into a microorganism having a β -carotene-synthesizing ability, culturing the transformed microorganism in a culture medium, and obtaining canthaxanthin or echinenone from the cultured cells.
29. A process for producing a xanthophyll comprising introducing the DNA strand according to any one of claims 10 - 18 into a microorganism having a zeaxanthin-synthesizing ability, culturing the transformed microorganism in a culture medium, and obtaining astaxanthin or 4-ketozeaxanthin from the cultured cells.
30. A process for producing a xanthophyll comprising introducing the DNA strand according to any one of claims 19 - 27 into a microorganism having a canthaxanthin-synthesizing ability, culturing the transformed microorganism in a culture medium, and obtaining astaxanthin or phoenicoxanthin from the cultured cells.
31. A process for producing a xanthophyll according to any one of claims 28 - 30, wherein the microorganism is a bacterium or yeast.

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237	246	255	264	273	282
GTG CAT GCG CTG TGG TTT CTG GAC GCA GCG GCG CAT CCC ATC CTG GCG ATC GCA					
Met His Ala Leu Trp Phe Leu Asp Ala Ala Ala His Pro Ile Leu Ala Ile Ala					
291	300	309	318	327	336
AAT TTC CTG GGG CTG ACC TGG CTG TCG GTC GGA TTG TTC ATC ATC GCG CAT GAC					
Asn Phe Leu Gly Leu Thr Trp Leu Ser Val Gly Leu Phe Ile Ile Ala His Asp					
345	354	363	372	381	390
GCG ATG CAC GGG TCG GTG GTG CCG GGG CGT CCG CGC GCC AAT GCG GCG ATG GGC					
Ala Met His Gly Ser Val Val Pro Gly Arg Pro Arg Ala Asn Ala Ala Met Gly					
399	408	417	426	435	444
CAG CTT GTC CTG TGG CTG TAT GCC GGA TTT TCG TGG CGC AAG ATG ATC GTC AAG					
Gln Leu Val Leu Trp Leu Tyr Ala Gly Phe Ser Trp Arg Lys Met Ile Val Lys					
453	462	471	480	489	498
CAC ATG GCC CAT CAC CGC CAT GCC GGA ACC GAC GAC GAC CCC GAT TTC GAC CAT					
His Met Ala His His Arg His Ala Gly Thr Asp Asp Asp Pro Asp Phe Asp His					
507	516	525	534	543	552
GGC GGC CCG GTC CGC TGG TAC GCC CGC TTC ATC GGC ACC TAT TTC GGC TGG CGC					
Gly Gly Pro Val Arg Trp Tyr Ala Arg Phe Ile Gly Thr Tyr Phe Gly Trp Arg					
561	570	579	588	597	606
GAG GGG CTG CTG CTG CCC GTC ATC GTG ACG GTC TAT GCG CTG ATC CTT GGG GAT					
Glu Gly Leu Leu Leu Pro Val Ile Val Thr Val Tyr Ala Leu Ile Leu Gly Asp					
615	624	633	642	651	660
CGC TGG ATG TAC GTG GTC TTC TGG CCG CTG CCG TCG ATC CTG GCG TCG ATC CAG					
Arg Trp Met Tyr Val Val Phe Trp Pro Leu Pro Ser Ile Leu Ala Ser Ile Gln					
669	678	687	696	705	714
CTG TTC GTG TTC GGC ACC TGG CTG CCG CAC CGC CCC GGC CAC GAC GCG TTC CCG					
Leu Phe Val Phe Gly Thr Trp Leu Pro His Arg Pro Gly His Asp Ala Phe Pro					
723	732	741	750	759	768
GAC CGC CAC AAT GCG CGG TCG TCG CGG ATC AGC GAC CCC GTG TCG CTG CTG ACC					
Asp Arg His Asn Ala Arg Ser Ser Arg Ile Ser Asp Pro Val Ser Leu Leu Thr					
777	786	795	804	813	822
TGC TTT CAC TTT GGC GGT TAT CAT CAC GAA CAC CAC CTG CAC CCG ACG GTG CCG					
Cys Phe His Phe Gly Gly Tyr His His Glu His His Leu His Pro Thr Val Pro					
831	840	849	858	867	
TGG TGG CGC CTG CCC AGC ACC CGC ACC AAG GGG GAC ACC GCA TGA					
Trp Trp Arg Leu Pro Ser Thr Arg Thr Lys Gly Asp Thr Ala ***					

↑
B

FIG. 1

C
↓

872	881	890	899	908	917
ATG ACC AAT TTC CTG ATC GTC GTC GCC ACC GTG CTG GTG ATG GAG TTG ACG GCC					
Met Thr Asn Phe Leu Ile Val Val Ala Thr Val Leu Val Met Glu Leu Thr Ala					
926	935	944	953	962	971
TAT TCC GTC CAC CGC TGG ATC ATG CAC GGC CCC CTG GGC TGG GGC TGG CAC AAG					
Tyr Ser Val His Arg Trp Ile Met His Gly Pro Leu Gly Trp Gly Trp His Lys					
980	989	998	1007	1016	1025
TCC CAC CAC GAG GAA CAC GAC CAC GCG CTG GAA AAG AAC GAC CTG TAC GGC CTG					
Ser His His Glu Glu His Asp His Ala Leu Glu Lys Asn Asp Leu Tyr Gly Leu					
1034	1043	1052	1061	1070	1079
GTC TTT GCG GTG ATC GCC ACG GTG CTG TTC ACG GTG GGC TGG ATC TGG GCG CCG					
Val Phe Ala Val Ile Ala Thr Val Leu Phe Thr Val Gly Trp Ile Trp Ala Pro					
1088	1097	1106	1115	1124	1133
GTC CTG TGG TGG ATC GCC TTG GGC ATG ACT GTC TAT GGG CTG ATC TAT TTC GTC					
Val Leu Trp Trp Ile Ala Leu Gly Met Thr Val Tyr Gly Leu Ile Tyr Phe Val					
1142	1151	1160	1169	1178	1187
CTG CAT GAC GGG CTG GTG CAT CAG CGC TGG CCG TTC CGT TAT ATC CCG CGC AAG					
Leu His Asp Gly Leu Val His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys					
1196	1205	1214	1223	1232	1241
GGC TAT GCC AGA CGC CTG TAT CAG GCC CAC CGC CTG CAC CAT GCG GTC GAG GGG					
Gly Tyr Ala Arg Arg Leu Tyr Gln Ala His Arg Leu His His Ala Val Glu Gly					
1250	1259	1268	1277	1286	1295
CGC GAC CAT TGC GTC AGC TTC GGC TTC ATC TAT GCG CCC CCG GTC GAC AAG CTG					
Arg Asp His Cys Val Ser Phe Gly Phe Ile Tyr Ala Pro Pro Val Asp Lys Leu					
1304	1313	1322	1331	1340	1349
AAG CAG GAC CTG AAG ATG TCG GGC GTG CTG CGG GCC GAG GCG CAG GAG CGC ACG					
Lys Gln Asp Leu Lys Met Ser Gly Val Leu Arg Ala Glu Ala Gln Glu Arg Thr					
TGA					

↑
D

FIG. 2

E



1357	1366	1375	1384	1393	1402
GTG ACC CAT GAC	GTG CTG CTG GCA GGG	GCG GGC CTT GCC	AAC GGG CTG ATC	GCC	
Met Thr His Asp	Val Leu Leu Ala Gly	Ala Gly Leu Ala Asn	Gly Leu Ile Ala		
1411	1420	1429	1438	1447	1456
CTG GCG CTG CGC	GCG GCG CGG CCC	GAC CTG CGC GTG	CTG CTG CTG GAC	CAT GGC	
Leu Ala Leu Arg	Ala Ala Arg Pro	Asp Leu Arg Val	Leu Leu Leu Asp	His Ala	
1465	1474	1483	1492	1501	1510
GCA GGA CCG TCA	GAC GGC CAC ACC	TGG TCC TGC CAC	GAC CCC GAC CTG	TCG CCG	
Ala Gly Pro Ser	Asp Gly His Thr	Trp Ser Cys His	Asp Pro Asp Leu	Ser Pro	
1519	1528	1537	1546	1555	1564
GAC TGG CTG GCG	CGG CTG AAG CCC	CTG CGC CGC GCC	AAC TGG CCC GAC	CAG GAG	
Asp Trp Leu Ala	Arg Leu Lys Pro	Leu Arg Arg Ala	Asn Trp Pro Asp	Gln Glu	
1573	1582	1591	1600	1609	1618
GTG CGC TTT CCC	CGC CAT GCC CGG	CGG CTG GCC ACC	GGT TAC GGG TCG	CTG GAC	
Val Arg Phe Pro	Arg His Ala Arg	Arg Leu Ala Thr	Gly Tyr Gly Ser	Leu Asp	
1627	1636	1645	1654	1663	1672
GGG GCG GCG CTG	GCG GAT GCG GTG	GTC CGG TCG GGC	GCC GAG ATC CGC	TGG GAC	
Gly Ala Ala Leu	Ala Asp Ala Val	Val Arg Ser Gly	Ala Glu Ile Arg	Trp Asp	
1681	1690	1699	1708	1717	1726
AGC GAC ATC GCC	CTG CTG GAT GCG	CAG GGG GCG ACG	CTG TCC TGC GGC	ACC CGG	
Ser Asp Ile Ala	Leu Leu Asp Ala	Gln Gly Ala Thr	Leu Ser Cys Gly	Thr Arg	
1735	1744	1753	1762	1771	1780
ATC GAG GCG GGC	GCG GTC CTG GAC	GGG CGG GGC GCG	CAG CCG TCG CGG	CAT CTG	
Ile Glu Ala Gly	Ala Val Leu Asp	Gly Arg Gly Ala	Gln Pro Ser Arg	His Leu	
1789	1798	1807	1816	1825	1834
ACC GTG GGT TTC	CAG AAA TTC GTG	GGT GTC GAG ATC	GAG ACC GAC CGC	CCC CAC	
Thr Val Gly Phe	Gln Lys Phe Val	Gly Val Glu Ile	Glu Thr Asp Arg	Pro His	
1843	1852	1861	1870	1879	1888
GGC GTG CCC CGC	CCG ATG ATC ATG	GAC GCG ACC GTC	ACC CAG CAG GAC	GGG TAC	
Gly Val Pro Arg	Pro Met Ile Met	Asp Ala Thr Val	Thr Gln Gln Asp	Gly Tyr	
1897	1906	1915	1924	1933	1942
CGC TTC ATC TAT	CTG CTG CCC TTC	TCT CCG ACG CGC	ATC CTG ATC GAG	GAC ACG	
Arg Phe Ile Tyr	Leu Leu Pro Phe	Ser Pro Thr Arg	Ile Leu Ile Glu	Asp Thr	
1951	1960	1969	1978	1987	1996
CGC TAT TCC GAT	GGC GGC GAT CTG	GAC GAC GCG CTG	GCG GCG GCG TCC	CAC	
Arg Tyr Ser Asp	Gly Gly Asp Leu	Asp Asp Ala Leu	Ala Ala Ala Ser	His	

FIG. 3

2005	2014	2023	2032	2041	2050
GAC TAT GCC CGC CAG CAG GGC TGG ACC GGG GCC GAG GTC CGG CGC GAA CGC GGC					
Asp Tyr Ala Arg Gln Gln Gly Trp Thr Gly Ala Glu Val Arg Arg Glu Arg Gly					
2059	2068	2077	2086	2095	2104
ATC CTT CCC ATC GCG CTG GCC CAT GAT GCG GCG GGC TTC TGG GCC GAT CAC GCG					
Ile Leu Pro Ile Ala Leu Ala His Asp Ala Ala Gly Phe Trp Ala Asp His Ala					
2113	2122	2131	2140	2149	2158
GCG GGG CCT GTT CCC GTG GGA CTG CGC GCG GGG TTC TTT CAT CCG GTC ACC GGC					
Ala Gly Pro Val Pro Val Gly Leu Arg Ala Gly Phe Phe His Pro Val Thr Gly					
2167	2176	2185	2194	2203	2212
TAT TCG CTG CCC TAT GCG GCA CAG GTG GCG GAC GTG GTG GCG GGT CTG TCC GGC					
Tyr Ser Leu Pro Tyr Ala Ala Gln Val Ala Asp Val Val Ala Gly Leu Ser Gly					
2221	2230	2239	2248	2257	2266
CCG CCC GGC ACC GAC GCG CTG CGC GGC GCC ATC CGC GAT TAC GCG ATC GAC CGG					
Pro Pro Gly Thr Asp Ala Leu Arg Gly Ala Ile Arg Asp Tyr Ala Ile Asp Arg					
2275	2284	2293	2302	2311	2320
GCG CGC CGC GAC CGC TTT CTG CGC CTT TTG AAC CGG ATG CTG TTC CGC GGC TGC					
Ala Arg Arg Asp Arg Phe Leu Arg Leu Leu Asn Arg Met Leu Phe Arg Gly Cys					
2329	2338	2347	2356	2365	2374
GCG CCC GAC CGG CGC TAT ACC CTG CTG CAG CGG TTC TAC CGC ATG CCG CAT GGA					
Ala Pro Asp Arg Arg Tyr Thr Leu Leu Gln Arg Phe Tyr Arg Met Pro His Gly					
2383	2392	2401	2410	2419	2428
CTG ATC GAA CGG TTC TAT GCC GGC CGG CTG AGC GTG GCG GAT CAG CTG CGC ATC					
Leu Ile Glu Arg Phe Tyr Ala Gly Arg Leu Ser Val Ala Asp Gln Leu Arg Ile					
2437	2446	2455	2464	2473	2482
GTG ACC GGC AAG CCT CCC ATT CCC CTT GGC ACG GCC ATC CGC TGC CTG CCC GAA					
Val Thr Gly Lys Pro Pro Ile Pro Leu Gly Thr Ala Ile Arg Cys Leu Pro Glu					
2491	2500	2509			
CGT CCC CTG CTG AAG GAA AAC GCA TGA					
Arg Pro Leu Leu Lys Glu Asn Ala ***					



F

FIG. 4

10	20	30	40	50	60
GGATC	CGGCG	ACCTT	GCGGC	GCTGC	GCCGC
CCTAG	GCCGC	TGGAA	CGCCG	CGACG	CGGCG
70	80	90	100	110	120
CAATG	GTCGC	AAGCA	ACGGG	GATGG	AAACC
GTTAC	CAGCG	TTCGT	TGCCC	CTACC	TTTGG
130	140	150	160	170	180
GCCGG	TCCGG	GGGAC	AAGAT	GAGCG	CACAT
CGGCC	AGGCC	CCCTG	TTCTA	CTCGC	GTGTA
190	200	210	220	A 230	240
AGCCT	GATCG	TCTCG	GGCGG	CATCA	TGCCC
TCGGA	CTAGC	AGAGC	CCGCC	GTAGT	AGCGG
250	260	270	280	290	300
TGGTT	TCTGG	ACGCA	GCGGC	GCATC	CCATC
ACCAA	AGACC	TGCGT	CGCCG	CGTAG	GGTAG
310	320	330	340	350	360
TGGCT	GTCGG	TCGGA	TTGTT	CATCA	TCGCG
ACCGA	CAGCC	AGCCT	AACAA	GTAGT	AGCGC
370	380	390	400	410	420
GGGCG	TCCGC	GCGCC	AATGC	GGCGA	TGGGC
CCCGC	AGGCG	CGCGG	TTACG	CCGCT	ACCCG
430	440	450	460	470	480
TCGTG	GCGCA	AGATG	ATCGT	CAAGC	ACATG
AGCAC	CGCGT	TCTAC	TAGCA	GTTCG	TGTAC
490	500	510	520	530	540
GACCC	CGATT	TCGAC	CATGG	CGGCC	CGGTC
CTGGG	GCTAA	AGCTG	GTACC	GCCGG	GCCAG
550	560	570	580	590	600
TTCGG	CTGGC	GCGAG	GGGCT	GCTGC	TGCCC
AAGCC	GACCG	CGCTC	CCCGA	CGACG	ACGGG
610	620	630	640	650	660
GGGGA	TCGCT	GGATG	TACGT	GGTCT	TCTGG
CCCCT	AGCGA	CCTAC	ATGCA	CCAGA	AGACC

FIG. 5

670	680	690	700	710	720
CTGTT CGTGT TCGGC ACCTG GCTGC CGCAC CGCCC CGGCC ACGAC GCGTT CCCGG ACCGC					
GACAA GCACA AGCCG TGGAC CGACG GCGTG GCGGG GCCGG TGCTG CGCAA GGGCC TGGCG					
730	740	750	760	770	780
CACAA TGCGC GGTCT TCGCG GATCA GCGAC CCCGT GTCGC TGCTG ACCTG CTTTC ACTTT					
GTGTT ACGCG CCAGC AGCGC CTAGT CGCTG GGGCA CAGCG ACGAC TGGAC GAAAG TGAAA					
790	800	810	820	830	840
GGCGG TTATC ATCAC GAACA CCACC TGCAC CCGAC GGTGC CGTGG TGGCG CCTGC CCAGC					
CCGCC AATAG TAGTG CTTGT GGTGG ACGTG GGCTG CCACG GCACC ACCGC GGACG GGTCG					
850	860	870	880	890	900
ACCCG CACCA AGGGG GACAC CGCAT GACCA ATTTT CTGAT CGTCG TCGCC ACCGT GCTGG					
TGGGC GTGGT TCCCC CTGTG GCGTA CTGGT TAAAG GACTA GCAGC AGCGG TGGCA CGACC					
910	920	930	940	950	960
TGATG GAGTT GACGG CCTAT TCCGT CCACC GCTGG ATCAT GCACG GCCCC CTGGG CTGGG					
ACTAC CTCAA CTGCC GGATA AGGCA GGTGG CGACC TAGTA CGTGC CGGGG GACCC GACCC					
970	980	990	1000	1010	1020
GCTGG CACAA GTCCC ACCAC GAGGA ACACG ACCAC GCGCT GGAAA AGAAC GACCT GTACG					
CGACC GTGTT CAGGG TGGTG CTCCT TGTGC TGGTG CGCGA CCTTT TCTTG CTGGA CATGC					
1030	1040	1050	1060	1070	1080
GCCTG GTCTT TGCGG TGATC GCCAC GGTGC TGTTC ACGGT GGGCT GGATC TGGGC GCCGG					
CGGAC CAGAA ACGCC ACTAG CCGTG CCACG ACAAG TGCCA CCCGA CCTAG ACCCG CGGCC					
1090	1100	1110	1120	1130	1140
TCCTG TGGTG GATCG CCTTG GGCAT GACTG TCTAT GGGCT GATCT ATTTC GTCCT GCATG					
AGGAC ACCAC CTAGC GGAAC CCGTA CTGAC AGATA CCCGA CTAGA TAAAG CAGGA CGTAC					
1150	1160	1170	1180	1190	1200
ACGGG CTGGT GCATC AGCGC TGGCC GTTCC GTTAT ATCCC GCGCA AGGGC TATGC CAGAC					
TGCCC GACCA CGTAG TCGCG ACCGG CAAGG CAATA TAGGG CGCGT TCCCG ATACG GTCTG					
1210	1220	1230	1240	1250	1260
GCCTG TATCA GGCCC ACCGC CTGCA CCATG CCGTC GAGGG GCGCG ACCAT TGCCT CAGCT					
CGGAC ATAGT CCGGG TGGCG GACGT GGTAC GCCAG CTCCC CGCGC TGGTA ACGCA GTCGA					
1270	1280	1290	1300	1310	1320
TCGGC TTCAAT CTATG CGCCC CCGGT CGACA AGCTG AAGCA GGACC TGAAG ATGTC GGGCG					
AGCCG AAGTA GATAC GCGGG GGCCA GCTGT TCGAC TTCGT CCTGG ACTTC TACAG CCCGC					

FIG. 6

1330	1340	E 1350	1360	1370	1380
TGCTG CGGGC CGAGG CCGAG GAGCG CACGT GACCC ATGAC GTGCT GCTGG CAGGG GCGGG	ACGAC GCCCC GCTCC GCGTC CTCGC GTGCA CTGGG TACTG CACGA CGACC GTCCC GCCCC				
1390	1400	1410	1420	1430	1440
CCTTG CCAAC GGGCT GATCG CCCTG GCGCT GCGCG CGGCG CGGCC CGACC TGC GC GTGCT	GGAAC GGTTC CCCGA CTAGC GGGAC CCGCA CGCGC GCCGC GCCGG GCTGG ACGCG CACGA				
1450	1460	1470	1480	1490	1500
GCTGC TGGAC CATGC CGCAG GACCG TCAGA CGGCC ACACC TGGTC CTGCC ACGAC CCCGA	CGACG ACCTG GTACG GCGTC CTGGC AGTCT GCCGG TGTGG ACCAG GACGG TGCTG GGGCT				
1510	1520	1530	1540	1550	1560
CCTGT CGCCG GACTG GCTGG CGCGG CTGAA GCCCC TGC GC CGCGC CAACT GGCCC GACCA	GGACA GCGGC CTGAC CGACC GCGCC GACTT CCGGG ACGCG GCGCG GTTGA CCGGG CTGGT				
1570	1580	1590	1600	1610	1620
GGAGG TGC GC TTTCC CCGCC ATGCC CGGCG GCTGG CCACC GGTTA CGGGT CGCTG GACGG	CCTCC ACGCG AAAGG GCGCG TACGG GCCGC CGACC GGTGG CCAAT GCCCA GCGAC CTGCC				
1630	1640	1650	1660	1670	1680
GGCGG CGCTG GCGGA TCGGG TGGTC CGGTC GGGCG CCGAG ATCCG CTGGG ACAGC GACAT	CCGCC GCGAC CGCCT ACGCC ACCAG GCCAG CCCGC GGCTC TAGGC GACCC TGTGC CTGTA				
1690	1700	1710	1720	1730	1740
CGCCC TGCTG GATGC GCAGG GGGCG ACGCT GTCCT GCGGC ACCCG GATCG AGGCG GCGCG	GCGGG ACGAC CTACG CGTCC CCCGC TCGCA CAGGA CGCCG TGGGC CTAGC TCCGC CCGCG				
1750	1760	1770	1780	1790	1800
GGTCC TGGAC GGGCG GGGCG CGCAG CCGTC GCGGC ATCTG ACCGT GGGTT TCCAG AAATT	CCAGG ACCTG CCCGC CCCGC GCGTC GGCAG CGCCG TAGAC TGGCA CCCAA AGGTC TTAA				
1810	1820	1830	1840	1850	1860
CGTGG GTGTC GAGAT CGAGA CCGAC CGCCC CCACG GCGTG CCCC GCGA TGATC ATGGA	GCACC CACAG CTCTA GCTCT GGCTG GCGGG GGTGC CGCAC GGGGC GGGCT ACTAG TACCT				
1870	1880	1890	1900	1910	1920
CGCGA CCGTC ACCCA GCAGG ACGGG TACCG CTTCA TCTAT CTGCT GCCCT TCTCT CCGAC	GCGCT GGCAG TGGGT CGTCC TGCCC ATGGC GAAGT AGATA GACGA CGGGA AGAGA GGCTG				
1930	1940	1950	1960	1970	1980
GCGCA TCCTG ATCGA GGACA CGCGC TATTC CGATG GCGGC GATCT GGACG ACGAC GCGCT	GCGCT AGGAC TAGCT CCTGT GCGCG ATAAG GCTAC CGCCG CTAGA CCTGC TGCTG CCGCA				

FIG. 7

1990			2000			2010			2020			2030			2040		
GGCGG	CGGCG	TCCCA	CGACT	ATGCC	CGCCA	GCAGG	GCTGG	ACCGG	GGCCG	AGGTC	CGGCG						
CCGCC	GCCGC	AGGGT	GCTGA	TACGG	GCGGT	CGTCC	CGACC	TGGCC	CCGGC	TCCAG	GCCGC						
2050			2060			2070			2080			2090			2100		
CGAAC	GCGGC	ATCCT	TCCCA	TCGCG	CTGGC	CCATG	ATGCG	GCGGG	CTTCT	GGGCC	GATCA						
GCTTG	CGCCG	TAGGA	AGGGT	AGCGC	GACCG	GGTAC	TACGC	CGCCC	GAAGA	CCCGG	CTAGT						
2110			2120			2130			2140			2150			2160		
CGCGG	CGGGG	CCTGT	TCCCG	TGGGA	CTGCG	CGCGG	GGTTC	TTTCA	TCCGG	TCACC	GGCTA						
GCGCC	GCCCC	GGACA	AGGGC	ACCCT	GACGC	GCGCC	CCAAG	AAAGT	AGGCC	AGTGG	CCGAT						
2170			2180			2190			2200			2210			2220		
TTCGC	TGCCC	TATGC	GGCAC	AGGTG	GCGGA	CGTGG	TGGCG	GGTCT	GTCCG	GGCCG	CCCGG						
AAGCG	ACGGG	ATACG	CCGTG	TCCAC	CGCCT	GCACC	ACCGC	CCAGA	CAGGC	CCGGC	GGGCC						
2230			2240			2250			2260			2270			2280		
CACCG	ACGCG	CTGCG	CGGCG	CCATC	CGCGA	TTACG	CGATC	GACCG	GGCGC	GCCGC	GACCG						
GTGGC	TGCGC	GACGC	GCCGC	GGTAG	GCGCT	AATGC	GCTAG	CTGGC	CCGCG	CGGCG	CTGGC						
2290			2300			2310			2320			2330			2340		
CTTTC	TGCGC	CTTTT	GAACC	GGATG	CTGTT	CCGCG	GCTGC	GCGCC	CGACC	GGCGC	TATAC						
GAAAG	ACGCG	GAAAA	CTTGG	CCTAC	GACAA	GGCGC	CGACG	CGCGG	GCTGG	CCGCG	ATATG						
2350			2360			2370			2380			2390			2400		
CCTGC	TGCAG	CGGTT	CTACC	GCATG	CCGCA	TGGAC	TGATC	GAACG	GTTCT	ATGCC	GGCCG						
GGACG	ACGTC	GCCAA	GATGG	CGTAC	GGCGT	ACCTG	ACTAG	CTTGC	CAAGA	TACGG	CCGGC						
2410			2420			2430			2440			2450			2460		
GCTGA	GCGTG	GCGGA	TCAGC	TGCGC	ATCGT	GACCG	GCAAG	CCTCC	CATTC	CCCTT	GGCAC						
CGACT	CGCAC	CGCCT	AGTCG	ACGCG	TAGCA	CTGGC	CGTTC	GGAGG	GTAAG	GGGAA	CCGTG						
2470			2480			2490			2500			2510			2520		
GGCCA	TCCGC	TGCCT	GCCCG	AACGT	CCCCT	GCTGA	AGGAA	AACGC	ATGAA	CGCCC	ATTGC						
CCGGT	AGGCG	ACGGA	CGGGC	TTGCA	GGGGA	CGACT	TCCTT	TTGCG	TACTT	GCGGG	TAAGC						
2530			2540			2550			2560			2570			2580		
CCCGC	GGCCA	AGACC	GCCAT	CGTGA	TCGGC	GCAGG	CTTTG	GCGGG	CTGGC	CCTGG	CCATC						
GGGCG	CCGGT	TCTGG	CGGTA	GCACT	AGCCG	CGTCC	GAAAC	CGCCC	GACCG	GGACC	GGTAG						
2590			2600			2610			2620			2630			2640		
CGCCT	GCAGT	CCGCG	GGCAT	CGCCA	CCACC	CTGGT	CGAGG	CCCGG	GACAA	GCCCC	GCGGG						
GCGGA	CGTCA	GGCGC	CCGTA	GCGGT	GGTGG	GACCA	GCTCC	GGGCC	CTGTT	CGGGC	CGCCC						

FIG. 8

2650	2660	2670	2680	2690	2700
CGCGC	CTATG	TCTGG	CACGA	TCAGG	GCCAT
CTCTT	CGACG	CGGGC	CCGAC	CGTCA	TCACC
GCGCG	GATAC	AGACC	GTGCT	AGTCC	CGGTA
GAGAA	GCTGC	GCCCG	GGCTG	GCACT	AGTGG
2710	2720	2730	2740	2750	2760
GACCC	CGATG	CGCTG	AAAGA	GCTGT	GGGCC
CTGAC	CGGGC	AGGAC	ATGGC	GCGCG	ACGTG
CTGGG	GCTAC	GCGAC	TTTCT	CGACA	CCC GG
GACTG	GCCCG	TCCTG	TACCG	GCGCG	TGCAC
2770	2780	2790	2800	2810	2820
ACGCT	GATGC	CGGTC	TCGCC	CTTCT	ATCGG
CTGAT	GTGGC	CGGGC	GGGAA	GGTCT	TCGAT
TGCGA	CTACG	GCCAG	AGCGG	GAAGA	TAGCC
GACTA	CACCG	GCCCG	CCCTT	CCAGA	AGCTA
2830	2840	2850	2860	2870	2880
TACGT	GAACG	AGGCC	GATCC	AGGGT	CTGGG
TCTTG	CCGTG	CCAGG	TGAAG	CTGTT	GCCGT
ATGCA	CTTGC	TCCGG	CTAGG	TCCCA	GACCC
AGAAC	GGCAC	GGTCC	ACTTC	GACAA	CGGCA
2886					
GGATC	C				
CCTAG	G				

FIG. 9



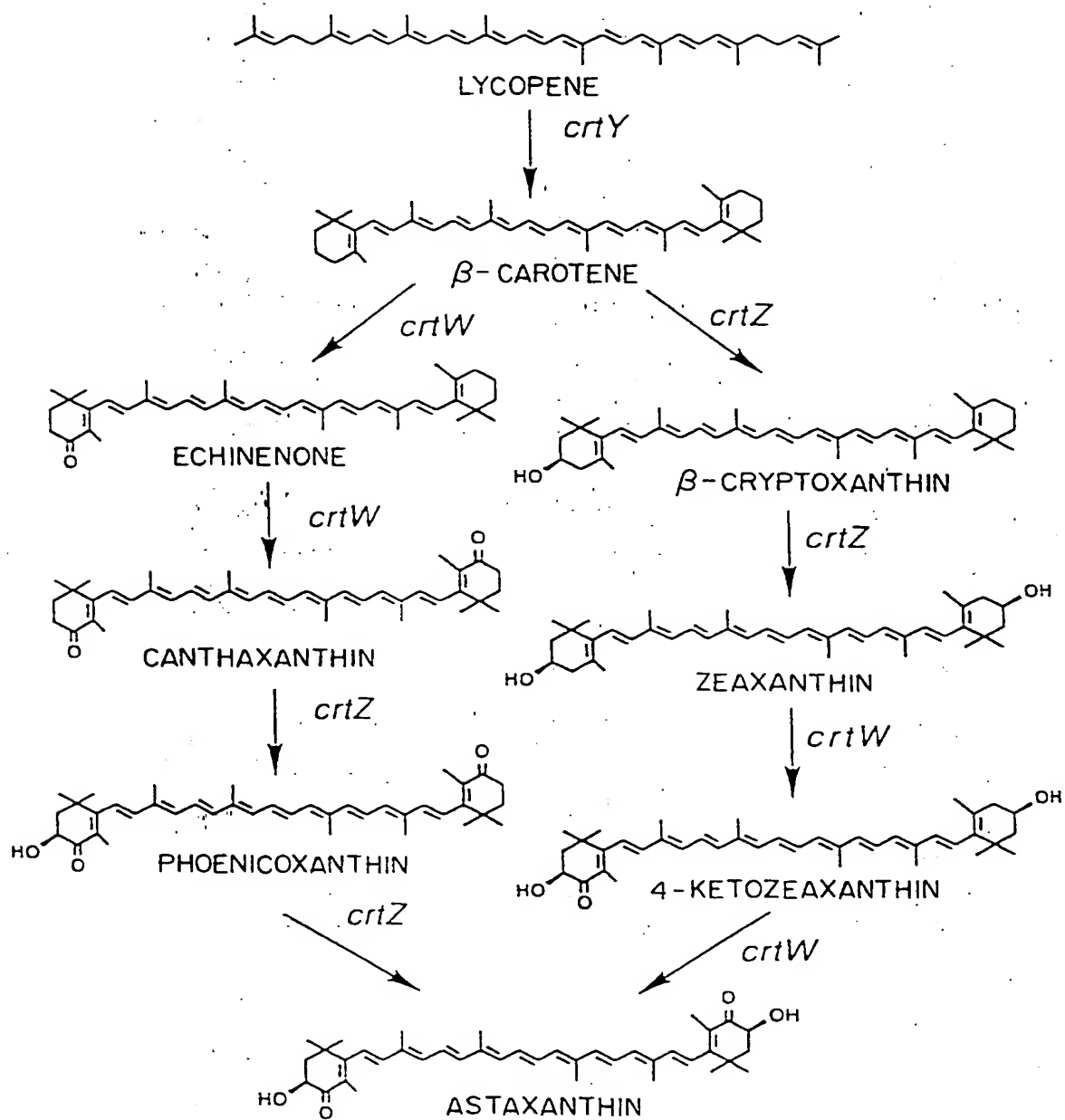


FIG. II

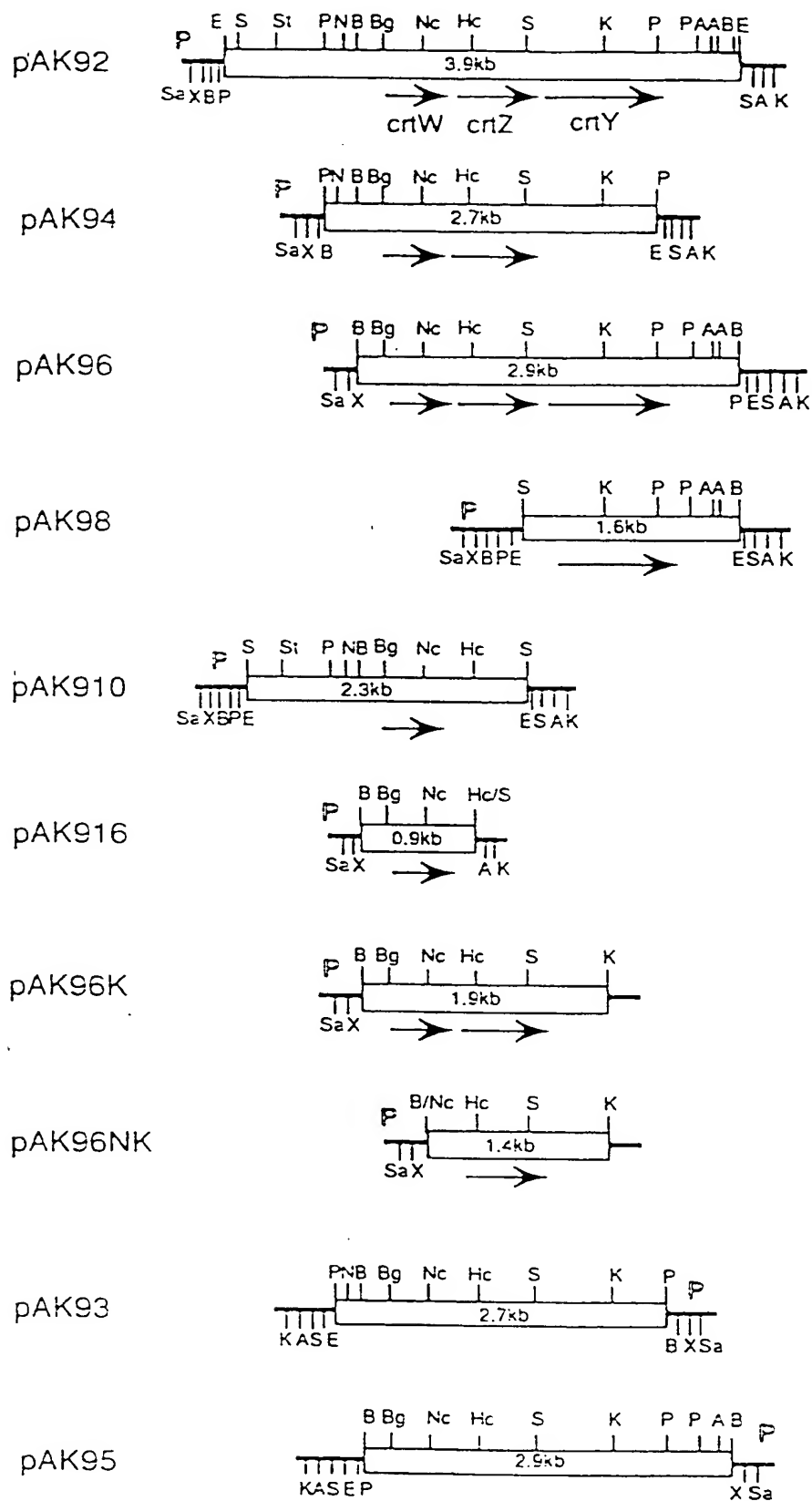


FIG. 12

A ↓
 110 120 130 140 150
 ATGTCCGGACGGAAGCCTGGCACAACCTGGCGACACGATCGTCAATCTCGGTCTGACCGCC
 1 MetSerGlyArgLysProGlyThrThrGlyAspThrIleValAsnLeuGlyLeuThrAla
 160 170 180 190 200 210
 GCGATCCTGCTGTGCTGGCTGGTCTGCACGCCTTTACGCTATGGTTGCTAGATGCGGCC
 21 AlaIleLeuLeuCysTrpLeuValLeuHisAlaPheThrLeuTrpLeuLeuAspAlaAla
 220 230 240 250 260 270
 GCGCATCCGCTGCTTGCCGTGCTGTGCCTGGCTGGGCTGACCTGGCTGTCTGGTCTGGGCTG
 41 AlaHisProLeuLeuAlaValLeuCysLeuAlaGlyLeuThrTrpLeuSerValGlyLeu
 280 290 300 310 320 330
 TTCATCATCGCGCATGACGCAATGCACGGGTCCGTGGTGCCGGGGCGGCCGCGCCCAAT
 61 PheIleIleAlaHisAspAlaMetHisGlySerValValProGlyArgProArgAlaAsn
 340 350 360 370 380 390
 GCGGCGATCGGGCAACTGGCGCTGTGGCTCTATGCGGGGTCTCGTGGCCCAAGCTGATC
 81 AlaAlaIleGlyGlnLeuAlaLeuTrpLeuTyrAlaGlyPheSerTrpProLysLeuIle
 400 410 420 430 440 450
 GCCAAGCACATGACGCATCACCGGCACGCCGGCACCGACAACGATCCCGATTTCGGTCCAC
 101 AlaLysHisMetThrHisHisArgHisAlaGlyThrAspAsnAspProAspPheGlyHis
 460 470 480 490 500 510
 GGAGGGCCCGTGCGCTGGTACGGCAGCTTCGTCTCCACCTATTTCCGGCTGGCGAGAGGGA
 121 GlyGlyProValArgTrpTyrGlySerPheValSerThrTyrPheGlyTrpArgGluGly
 520 530 540 550 560 570
 CTGCTGCTACCGGTGATCGTCACCACCTATGCGCTGATCCTGGGCGATCGCTGGATGTAT
 141 LeuLeuLeuProValIleValThrThrTyrAlaLeuIleLeuGlyAspArgTrpMetTyr
 580 590 600 610 620 630
 GTCATCTTCTGGCCGGTCCCGGCCGTCTTGGCGTCGATCCAGATTTTCGTCTTCGGAACT
 161 ValIlePheTrpProValProAlaValLeuAlaSerIleGlnIlePheValPheGlyThr
 640 650 660 670 680 690
 TGGCTGCCCCACCGCCCGGGACATGACGATTTTCCCGACCGGCACAACGCGAGGTGCGACC
 181 TrpLeuProHisArgProGlyHisAspAspPheProAspArgHisAsnAlaArgSerThr
 700 710 720 730 740 750
 GGCATCGGCGACCCGTTGTCACTACTGACCTGCTTCCATTTCCGGCGGCTATCACCACGAA
 201 GlyIleGlyAspProLeuSerLeuLeuThrCysPheHisPheGlyGlyTyrHisHisGlu

FIG. 13

760 770 780 790 800 810
CATCACCTGCATCCGCATGTGCCGTGGTGGCGCCTGCCTCGTACACGCAAGACCGGAGGC
221 HisHisLeuHisProHisValProTrpTrpArgLeuProArgThrArgLysThrGlyGly

820 827
CGCGCATGA
241 ArgAla***

↑B

FIG. 14

C↓
 830 840 850 860 870 880
 ATGACGCAATTCCTCATTGTCGTGGCGACAGTCCTCGTGATGGAGCTGACCGCCTATTCC
 1 MetThrGlnPheLeuIleValValAlaThrValLeuValMetGluLeuThrAlaTyrSer
 890 900 910 920 930 940
 GTCCACCGCTGGATTATGCACGGCCCCCTAGGCTGGGGCTGGCACAAGTCCCATCACGAA
 21 ValHisArgTrpIleMetHisGlyProLeuGlyTrpGlyTrpHisLysSerHisHisGlu
 950 960 970 980 990 1000
 GAGCACGACCACGCGTTGGAGAAGAACGACCTCTACGGCGTCGTCTTCGCGGTGCTGGCG
 41 GluHisAspHisAlaLeuGluLysAsnAspLeuTyrGlyValValPheAlaValLeuAla
 1010 1020 1030 1040 1050 1060
 ACGATCCTCTTCACCGTGGGCGCCTATTGGTGGCCGGTGCTGTGGTGGATCGCCCTGGGC
 61 ThrIleLeuPheThrValGlyAlaTyrTrpTrpProValLeuTrpTrpIleAlaLeuGly
 1070 1080 1090 1100 1110 1120
 ATGACGGTCTATGGGTTGATCTATTTTCATCCTGCACGACGGGCTTGTGCATCAACGCTGG
 81 MetThrValTyrGlyLeuIleTyrPheIleLeuHisAspGlyLeuValHisGlnArgTrp
 1130 1140 1150 1160 1170 1180
 CCGTTTCGGTATATTCCGCGCGGGGCTATTTCCGCAGGCTCTACCAAGCTCATCGCCTG
 101 ProPheArgTyrIleProArgArgGlyTyrPheArgArgLeuTyrGlnAlaHisArgLeu
 1190 1200 1210 1220 1230 1240
 CACCACGTCGGTCGAGGGGCGGGACCACTGCGTCAGCTTCGGCTTCATCTATGCCCCACCC
 121 HisHisAlaValGluGlyArgAspHisCysValSerPheGlyPheIleTyrAlaProPro
 1250 1260 1270 1280 1290 1300
 GTGGACAAGCTGAAGCAGGATCTGAAGCGGTGCGGTGTCCTGCGCCCCCAGGACGAGCGT
 141 ValAspLysLeuLysGlnAspLeuLysArgSerGlyValLeuArgProGlnAspGluArg
 1312
 CCGTCGTGA
 161 ProSer***

↑ D

FIG. 15

10	20	30	40	50	60
CTGCA	GGCCG	GGCCC	GGTGG	CCAAT	GGTCG
GACGT	CCGGC	CCGGG	CCACC	GGTTA	CCAGC
					GGTGG
					CCGTC
					CTGAC
					CTTGT
					CCTGC
					CGCCC
70	80	90		110	120
CCGGT	CTAGG	CTGTC	GCCCT	ACGCA	GCAGG
GGCCA	GATCC	GACAG	CGGGA	TGCGT	CGTCC
					TCAAA
					GCCTA
					CAGGC
					CTGCC
					TTCGG
					ACCGT
130	140	150	160	170	180
CAACT	GGCGA	CACGA	TCGTC	AATCT	CGGTC
GTTGA	CCGCT	GTGCT	AGCAG	TTAGA	GCCAG
					ACTGG
					CGGCG
					CTAGG
					ACGAC
					ACGAC
					CGACC
190	200	210	220	230	240
TCCTG	CACGC	CTTTA	CGCTA	TGGTT	GCTAG
AGGAC	GTGCG	GAAAT	GCGAT	ACCAA	CGATC
					TACGC
					CGGCG
					CGTAG
					GCGAC
					GAACG
					GCACG
250	260	270	280	290	300
TGTGC	CTGGC	TGGGC	TGACC	TGGCT	GTCGG
ACACG	GACCG	ACCCG	ACTGG	ACCGA	CAGCC
					AGCCC
					GACAA
					GTAGT
					AGCGC
					GTAAT
					GCGCT
310	320	330	340	350	360
TGCAC	GGGTC	CGTGG	TGCCG	GGGCG	GCCGC
ACGTG	CCCAG	GCACC	ACGGC	CCCGC	CGGCG
					CGCGG
					TTACG
					CCGCT
					AGCCC
					GTTGA
					CCGCG
370	380	390	400	410	420
TGTGG	CTCTA	TGCGG	GGTTC	TCGTG	GCCCA
ACACC	GAGAT	ACGCC	CCAAG	AGCAC	CGGGT
					TCGAC
					TAGCG
					GTTCT
					TGTAC
					TGCGT
					AGTGG
430	440	450	460	470	480
GGCAC	GCCGG	CACCG	ACAAC	GATCC	CGATT
CCGTG	CGGCC	GTGGC	TGTTG	CTAGG	GCTAA
					AGCCA
					GTGCC
					TCCCG
					GGCAC
					GCGAC
					CATGC
490	500	510	520	530	540
GCAGC	TTCGT	CTCCA	CCTAT	TTCGG	CTGGC
CGTCG	AAGCA	GAGGT	GGATA	AAGCC	GACCG
					CTCTC
					CCTGA
					CGACG
					ATGGC
					CACTA
					GCAGT
550	560	570	580	590	600
CCACC	TATGC	GCTGA	TCCTG	GGCGA	TCGCT
GGTGG	ATACG	CGACT	AGGAC	CCGCT	AGCGA
					CCTAC
					ATACA
					GTAGA
					AGACC
					GGCCA
					GGGCC
610	620	630	640	650	660
CCGTT	CTGGC	GTCGA	TCCAG	ATTTT	CGTCT
GGCAA	GACCG	CAGCT	AGGTC	TAAAA	GCAGA
					AGCCT
					TGAAC
					CGACG
					GGGTG
					GCGGG
					CCCTG
670	680	690	700	710	720
ATGAC	GATTT	TCCCG	ACCGG	CACAA	CGCGA
TACTG	CTAAA	AGGGC	TGGCC	GTGTT	GCGCT
					CCAGC
					TGGCC
					GTAGC
					CGCTG
					GGCAA
					CAGTG

FIG. 16

730	740	750	760	770	780
TACTG	ACCTG	CTTCC	ATTTT	GGCGG	CTATC
ATGAC	TGGAC	GAAGG	TAAAG	CCGCC	GATAG
790	800	810	820	830	840
CGTGG	TGGCG	CCTGC	CTCGT	ACACG	CAAGA
GCACC	ACCGC	GGACG	GAGCA	TGTGC	GTTCT
850	860	870	880	890	900
TGTCG	TGGCG	ACAGT	CCTCG	TGATG	GAGCT
ACAGC	ACCGC	TGTCA	GGAGC	ACTAC	CTCGA
910	920	930	940	950	960
GCACG	GCCCC	CTAGG	CTGGG	GCTGG	CACAA
CGTGC	CGGGG	GATCC	GACCC	CGACC	GTGTT
970	980	990	1000	1010	1020
GGAGA	AGAAC	GACCT	CTACG	GCGTC	GTCTT
CCTCT	TCTTG	CTGGA	GATGC	CGCAG	CAGAA
1030	1040	1050	1060	1070	1080
GGGCG	CCTAT	TGGTG	GCCGG	TGCTG	TGGTG
CCCGC	GGATA	ACCAC	CGGCC	ACGAC	ACCAC
1090	1100	1110	1120	1130	1140
GATCT	ATTTT	ATCCT	GCACG	ACGGG	CTTGT
CTAGA	TAAAG	TAGGA	CGTGC	TGCC	GAACA
1150	1160	1170	1180	1190	1200
GCGGC	GGGGC	TATTT	CCGCA	GGCTC	TACCA
CGCCG	CCCCG	ATAAA	GGCGT	CCGAG	ATGGT
1210	1220	1230	1240	1250	1260
GCGGG	ACCAC	TGCGT	CAGCT	TCGGC	TTCAT
CGCCC	TGGTG	ACGCA	GTCGA	AGCCG	AAGTA
1270	1280	1290	1300	1310	1320
GGATC	TGAAG	CGGTC	GGGTG	TCCTG	CGCCC
CCTAG	ACTTC	GCCAG	CCCAC	AGGAC	GCGGG
1330	1340	1350	1360	1370	1380
CCCGG	CGTGG	CCGCA	TGAAA	TCCGA	CGTGC
GGGCC	GCACC	GGCGT	ACTTT	AGGCT	GCACG
1390	1400	1410	1420	1430	1440
TGATC	GCGCT	GGCGA	TCCGC	AAGGC	GCGGC
ACTAG	CGCGA	CCGCT	AGGCG	TTCGG	CGCCC

C↓

↑B

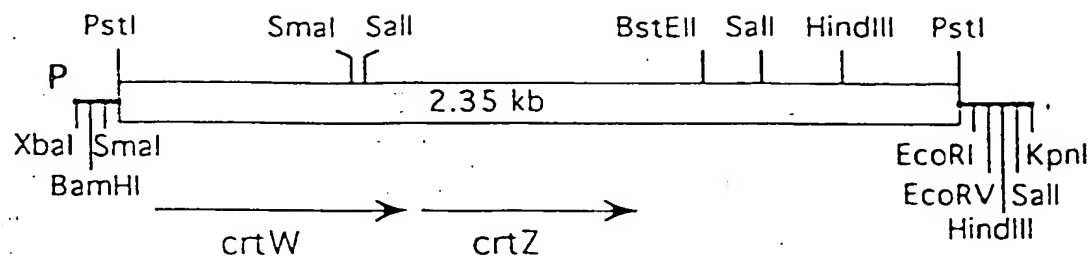
↑D

FIG. 17

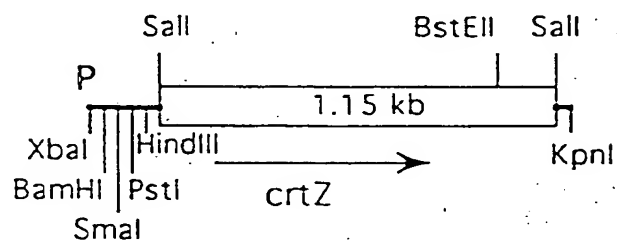
1450	1460	1470	1480	1490	1500
CGGCG GCGGC CTCGG	ACGGG CATA	TTGGT CCTGC	CACGA CACCG	ATTTG GCGCC	GCACT
GCCGC CCGCG	GAGCC TGCCC	GTATG AACCA	GGACG GTGCT	GTGGC TAAAC	CGCGG CGTGA
1510	1520	1530	1540	1550	1560
GGCTG GACCG	CCTGA AGCCG	ATCAG GCGTG	GCGAC TGGCC	CGATC AGGAG	GTGCG GTTCC
CCGAC CTGGC	GGACT TCGGC	TAGTC CGCAC	CGCTG ACCGG	GCTAG TCCTC	CACGC CAAGG
1570	1580	1590	1600	1610	1620
CAGAC CATT	C GCGAA	GGCTC CGGGC	CGGAT ATGGC	TCGAT CGACG	GGCGG GGGCT
GTCTG GTAAG	CGCTT CCGAG	GCCCC GCCTA	TACCG AGCTA	GCTGC CCGCC	CCCGA CTACG
1631					
GTGCG GTGAC	C				
CACGC CACTG	G				

FIG. 18

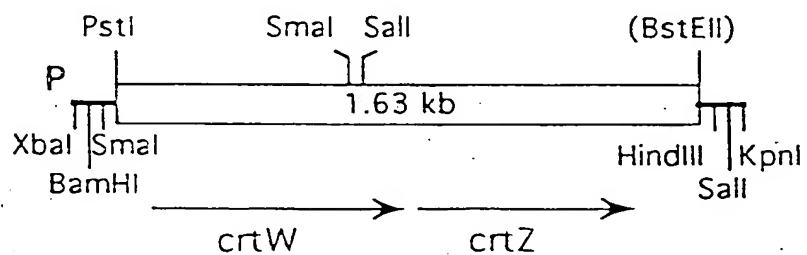
pPC11



pPC13



pPC17



pPC17-3

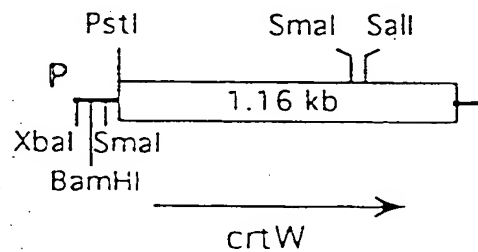


FIG. 19

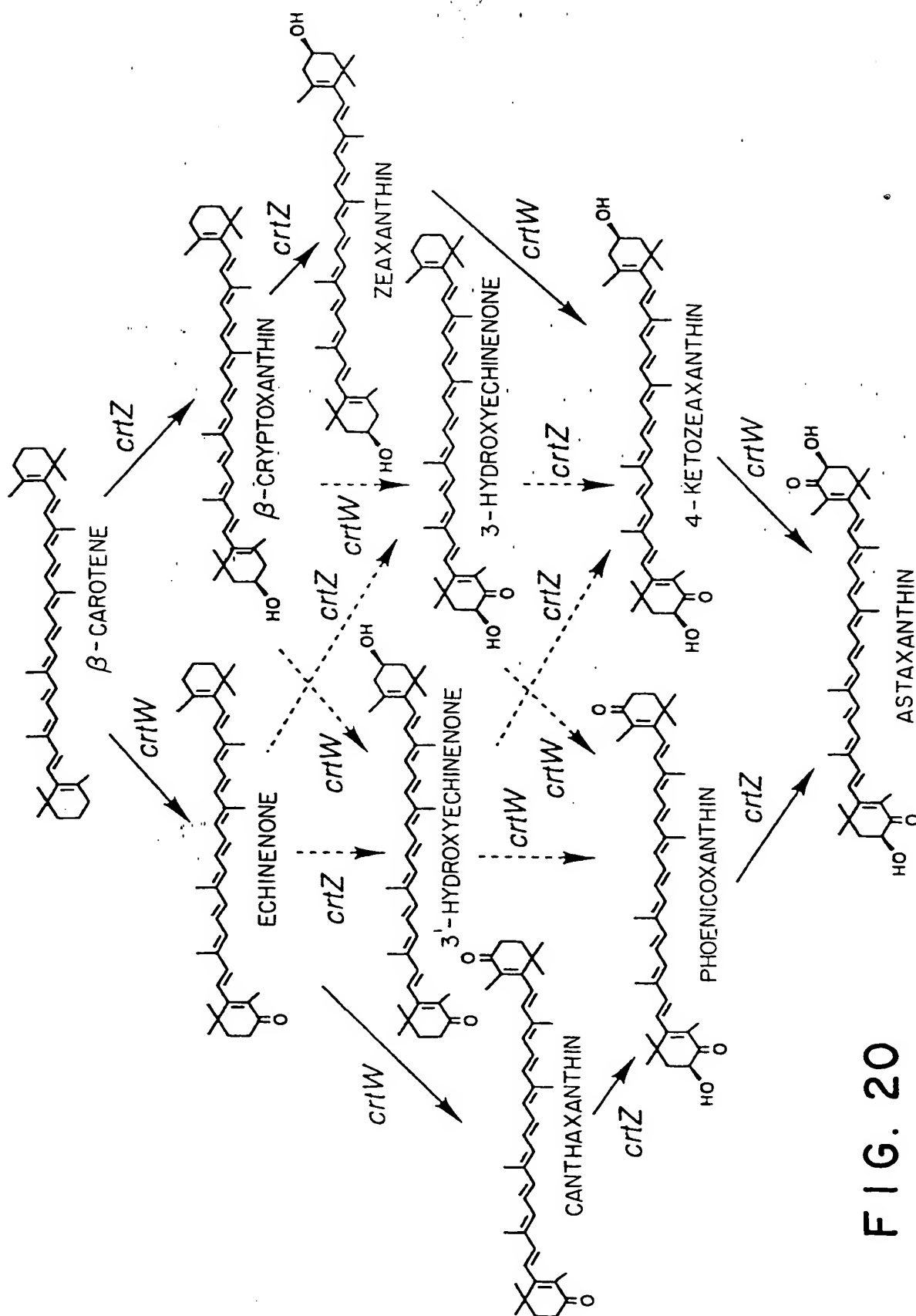


FIG. 20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP94/02220

A. CLASSIFICATION OF SUBJECT MATTER

Int. C16 C12N15/00, C12P7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. C16 C12N15/00, C12P7/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS, WPI/WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO, A, 9406918 (Gist-Brocades NV.), March 31, 1994 (31. 03. 94) & EP, A, 586751 & CA, A, 2105957	1-31
A	EP, A, 474347 (Unilever Plc, Quest Int. BV.), March 11, 1992 (11. 03. 92) & JP, A, 5-076347	1-31
A	"Marine bacteria produced astaxanthin" 10th International symposium on carotenoids, abstract, CL11-3(1993)	1-31

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Date of the actual completion of the international search

March 16, 1995 (16. 03. 95)

Date of mailing of the international search report

April 4, 1995 (04. 04. 95)

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